Docket No. PF112P1D2

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of: Hu et al.

Application No.: 10/023,584 Group Art Unit: 1647

Filed: December 21, 2001 Examiner: R. Landsman

For: Antibodies to Human Endothelial Growth Factor 2 (as amended herein)

Declaration of Viktor Roschke under 37 C.F.R. § 1.132

Sir:

I, Viktor Roschke, Ph.D., hereby declare and state as follows:

I. Biographical Information

I am currently employed as the Director of Clinical Immunology and Antibody Development at Human Genome Sciences, Inc. (HGS), which I understand to be the assignee of the above-captioned patent application. I received my M.S. degree in Biochemistry in 1977 from the Novosibirsk University. I earned my Ph.D. in 1985 from the Novosibirsk Institute of Bioorganic Chemistry of the Russian Academy of Sciences after completion of 5 years of research in the field of immunochemical analysis of biomolecules. This research entailed extensive antibody development work. From 1985 to 1992, I conducted independent research in monoclonal antibody development and applications of immunochemical techniques to understanding the structure and function of biomolecules. I was also significantly involved in implementation of new immunochemical techniques for use in medical diagnostics. In 1992, I joined the Laboratory of Genetics at National Cancer Institute (NCI) in Bethesda, Maryland. I spent 5 years at NCI studying the biology of antibody-producing B cell tumors known as plasmacytomas. In 1997, I joined Human Genome Sciences where my research work included, in large part, both carrying out and supervising the development and characterization of therapeutic and reagent antibodies in support of pre-clinical and clinical research on protein and antibody-based therapeutics. I have co-authored

VR 12 Apr04

a book chapter and ~~36~~ 37 scientific articles that have been published in peer-reviewed scientific journals. A copy of my curriculum vitae is attached as Exhibit 1.

I have been asked by patent counsel for HGS to provide my understanding of the meaning of the phrase "specifically binds" in the context of antibodies that specifically bind a particular protein (*e.g.*, "an antibody that specifically binds Protein-X"), as this phrase was understood by researchers experienced in using antibodies on or before March 8, 1994. Furthermore, although this declaration addresses the perspective of researchers on or before March 8, 1994, it should also be emphasized that researchers' current understanding of the phrase "specifically binds" (in the context of antibodies) remains consistent with the meaning of this phrase as described herein.

II. Meaning of "Specifically Binds"

A. In order to discuss the meaning of the phrase "an antibody that specifically binds" a particular protein, one must understand the related concept of "antibody specificity." The specificity of an antibody refers to its capacity to discriminate between antigens of different structure. An antibody that is "specific" or has "specificity" for a particular antigen can also be said to be "an antibody that specifically binds" that antigen. The fact that antibodies have "specificity" has made them invaluable research tools. Often, a scientist seeks to isolate antibodies that are specific for a particular antigen which they would like to study further. This is done by immunizing an animal (or screening an antibody expression library, such as a phage display or Fab library) with that antigen in order to identify and isolate an antibody that is "specific" for the antigen with which the animal was immunized (or with which the library was screened).

B. Antibodies can also be described in terms of their "cross-reactivity." Thus, antibodies are commonly characterized as "specific" or "cross-reactive" based on an evaluation of their specificity. A specific antibody (*i.e.*, an antibody with specificity) binds the antigen against which it was raised/screened (the target antigen), but does not bind closely related antigens of a different structure. In contrast, a cross-reactive antibody may bind the target antigen against which it was raised/screened, but also binds antigens with structure

differing from the target antigen. *See e.g.*, *Immunology: a Synthesis* by Edward Golub and Douglas Green (2nd edition, Sunderland, MA: Sinauer Associates, Inc.1991:p. 27, attached herewith as Exhibit 2).

C. Specificity and cross-reactivity, however, are not mutually exclusive terms. A single antibody can be described in terms of both the protein(s) for which it is "specific" and/or the protein(s) with which it cross-reacts. Specific binding focuses on the antibody's ability to bind the antigen against which it was raised/screened, whereas cross-reactive binding focuses on the antibody's ability to bind antigens different from the antigen against which it was raised/screened. Hence, the concept of "specificity" encompasses the concepts of specific binding and cross-reactive binding.

Stated another way, a "specific antibody" is one that is discriminating in that it preferentially binds the antigen against which it was raised/screened, compared to its ability to bind other antigens. A "cross-reactive antibody" is one that is not discriminating in that it does not show a strong preference for binding the antigen against which it was raised/screened compared to its ability to bind other antigens. Thus, a "specific antibody" binds the antigen against which it was raised/screened with significantly higher affinity than it binds other antigens. Conversely, a "cross-reactive antibody" binds the antigen against which it was raised/screened with an affinity comparable to that with which it binds other antigens. Thus, a cross-reactive antibody is less useful for discriminating target from non-target antigens.

D. Within an organism, an antibody's functional ability depends on its specificity. Thus, in an immune response antibodies must be specific *(e.g.,* able to discriminate between the infecting pathogen and the host), otherwise the immune system would not be able to eliminate pathogens while sparing the host. Hence, antibodies that are "specific" are essential because they can target (*i.e.*, bind to) a particular antigen within a population of heterogenous antigens.

E. Antibody specificity is an important characteristic of an antibody, since as mentioned above, it is their specificity - their ability to detect a given antigen in the presence

of other antigens - that makes antibodies useful as agents of the immune system, as tools in biological assays, and as therapeutic agents.

III. Specifically Binds As Understood by Those Using Antibodies in Research

A. On and before March 8, 1994, those who ordinarily practiced research with antibodies (for example, those who practiced research in areas such as immunology and cell & molecular biology) would routinely use the phrase "specifically binds" to refer to the functional ability of an antibody to preferentially bind a particular (target) antigen instead of a non-target antigen. Thus, it was understood that an antibody that "specifically bound" a target antigen was an antibody with "specificity for" that target antigen. As an example, the textbook *Immunology: a Synthesis* by Edward Golub and Douglas Green (2nd edition, Sunderland, MA: Sinauer Associates, Inc.1991:p. 23, attached herewith as Exhibit 2) defined specificity as "the ability of antibodies produced in response to an antigen to react with that antigen and not with others."

Thus, an antibody that specifically bound Protein-X (the target antigen) was understood to be an antibody that preferentially bound Protein-X without a significant level of binding to other proteins (*i.e.*, without a significant level of "cross-reactivity" or "cross-reaction"). In this context "preferentially bound" "without a significant level of binding" to other antigens was understood to mean that the antibody's ability to specifically bind the target antigen made it useful for discriminating the target antigen from other antigens in biological assays, diagnostic assays, or treatment protocols (such as in cell staining, immunoblot analyses, immunoprecipitations, enzyme-linked immunosorbent assays (ELISA), or *in vivo* binding of the target antigen).

B. However, it was also understood that an antibody that specifically bound Protein-X but did not cross-react with other antigens was not necessarily an antibody that could not bind other antigens under all conditions. Indeed, those who ordinarily practiced research with antibodies understood (and still understand) that antibodies could (and can) be made to bind non-target antigens by utilization of non-optimal antibody binding conditions. For example, use of non-optimal antibody, antigen, total protein, salt, and/or detergent

concentrations was (and is) known to produce non-specific antibody binding by antibodies that would, otherwise, be considered "specific." Importantly, those who ordinarily practiced research with antibodies on and before March 8, 1994 routinely optimized antibody binding conditions (as is done by practitioners to date). Thus, the ability of a Protein-X specific antibody to bind Protein-X was (and is) compared to its inability to bind non-Protein-X antigens under empirically optimized conditions established by routine manipulation of antibody binding conditions. Thus, it was understood that an antibody that specifically bound Protein-X could be recognized by its usefulness in at least one type of biological assay, diagnostic assay, or treatment protocol because of the insignificant background levels or lack of adverse effects resulting from antibody binding to non-Protein-X antigens.

C. Additionally, the fact that an antibody specifically bound a polypeptide in one species (*e.g.*, human) and also bound the orthologous polypeptide in another species (*e.g.*, mouse)[1] would not change an immunologists characterization of that antibody as "specific." Hence, it was understood that an antibody that specifically bound human Protein-X could also specifically bind murine Protein-X. Such cross-species binding was (and is) merely understood to indicate that the antigenic determinant to which the Protein-X antibody binds is conserved in both the human and mouse Protein-X orthologs. In contrast, an antibody that bound equally well to human Protein-X and human Protein-Y (*i.e.*, paralogous polypeptides) would not be an antibody that an immunologist would have considered as one that "specifically" bound Protein-X. Consider, for example, the 1994 *Boehringer Mannheim*

[1]The terms "orthologous" and "ortholog/orthologue" versus "paralogous" and "paralog/paralogue" are used herein as these terms are generally understood and in accord with a definition provided by the National Center for Biotechnology (NCBI). *See* the NCBI Field Glossary available on the internet at http://www.ncbi.nlm.nih.gov/Class/FieldGuide/glossary.html#A, a printout of the excerpted definitions for orthologue and paralogue are attached herewith as Exhibit 3. Orthologous genes (or, genes which are orthologues) are derived from a common ancestor through vertical evolutionary descent. Thus, genes (and proteins encoded by them) are considered to be orthologues when they represent the *same* gene (or protein) found in *different* species. For example, feline FGF-1 (fibroblast growth factor-1), murine FGF-1, and human FGF-1 are orthologues (orthologous proteins). In contrast, paralogous genes (or, genes which are paralogues) are genes found within the same genome that are thought to have evolved by gene duplication. For example, human FGF-1, human FGF-2, and human FGF-3 are gene paralogues; and, the proteins encoded by these genes are also paralogues (or paralogous proteins).

Biochemicals Catalog which used the term "specifically" to describe an antibody that bound both murine and human Mac-1 protein. In particular, the *Boehringer Catalog* listed an Anti-Mac-1 (macrophage associated antigen) (clone M1/70) antibody and described it under the heading "Specificity and Notes":

> The antibody specifically reacts with native mouse and human Mac-1 (complement receptor type 3; Ly-40) antigen and precipitates two chains, 170kD (CD11b) and 95kD (CD18).

See, Boehringer Mannheim Biochemicals, Inc. 1994 Catalog, page 260, enclosed herewith as part of Exhibit 4 (emphasis added). Thus, providing a particular example of an antibody described as "specifically" binding orthologous proteins in mouse and human species.

D. Those who routinely used antibodies in research also understood that an antibody that "specifically bound" Protein-X might also be capable of binding Protein-X in a variety of forms. Hence, an antibody that specifically bound Protein-X might also be expected to bind Protein-X in either denatured or native forms. For example, the 1994 *Boehringer Catalog* listed Anti-GABA receptor, α-chain and β-chain antibodies as useful for both western blots (denatured protein) and immunoprecipitation assays (native protein). *See, Boehringer Catalog*, pages 280 and 281, enclosed herewith as part of Exhibit 4.

Additionally, depending on the presence or absence of the Protein-X antigenic epitope to which the antibody binds, an antibody that specifically bound Protein-X could also bind fragments or variants of Protein-X, such as Protein-X orthologues, Protein-X splice variants, and Protein-X allelic variants (*i.e.*, polypeptides encoded by mutated forms of Gene X). For example, the 1994 *Boehringer Catalog* listed and described an Anti-L-CAM/Uvomorulin (clone 6F9) antibody as one that specifically bound 120kD and 80kD forms of L-CAM/Uvomorulin:

> The antibody specifically recognizes the 120 kD and the 80 kD band of L-CAM/Uvomorulin (Arc-1 E-cadherin cell-CAM 120/80) in man and rabbit. L-CAM/Uvomorulin staining is confined to the lateral border of epithelial cells and, within the intestine, shows more intense concentrations in the area of the junctional complex. As a positive control, the cell line MCF-7 can be used.

See, Boehringer Catalog, page 280, attached hereto as part of Exhibit 4 (emphasis added).

Notably, the *Boehringer Catalog* (*supra*) did not state or imply that the "specific" antibodies listed bound the desired protein to the exclusion of all other proteins. However, it would have been clear to those who routinely used antibodies in research that these antibodies preferentially bound their target antigen to the exclusion of non-related proteins because an antibody that was not specific (*i.e.*, did not distinguish between the protein of interest and other non-related proteins) would not have been useful, for example, in immunoassays, purification processes, or disease treatment regimens.

IV. Determining Specificity

A. On and before March 8, 1994, the description of antibodies as "specifically binding" a particular antigen was routinely used, recognized, and understood by those who ordinarily practiced research with antibodies. Additionally, methods for determining antibody specificity were also routine and well known on and before March 8, 1994. For example, routine assays for identifying a "specific" antibody were described in *Current Protocols in Immunology*, a common laboratory handbook. More particularly, *Current Protocols* provided examples of three such assays: (1) Indirect ELISA to Detect *Specific* Antibodies; (2) Double Antibody-Sandwich ELISA to Detect *Specific* Antibodies; and (3) Double-Immunodiffusion Assay for Detecting *Specific* Antibodies (*see, e.g., Current Protocols in Immunology* ed. Coligan *et al.* Vol. 2, Sections 2.1.1-2.1.20 and 2.3.1-2.3.4 (1991), attached hereto as Exhibit 5 (emphasis added).

B. Experimentally, antibody specificity was (and is) determined using assays, such as those indicated above, to establish that an antibody binds directly and preferentially to the target antigen, but not to other antigens. It was also known that antibody specificity could be further confirmed by showing that soluble target antigen competitively inhibited antibody binding in a concentration dependent manner; but that other antigens did not competitively inhibit antibody binding to the target antigen. Thus, in an ELISA assay, Protein-X-specific antibodies would preferentially bind a plate coated with Protein-X compared to its ability to bind to an ELISA plate coated with Protein-Y Moreover, specificity could (and can) also be tested by showing that antibody binding to Protein-X (by a Protein-X-

specific antibody) could be competitively inhibited by soluble Protein-X (but not by soluble ProteinY) in a concentration dependent manner.

C. The data shown in Exhibit 6 demonstrates many of the points explained above. The results shown in Exhibit 6 were obtained using an ELISA assay. ELISA assays were routinely used on or before March 8, 1994, as they are to date, to analyze antibody binding specificity. Exhibit 6 shows antibody binding results obtained with polyclonal antibodies that specifically bind the target antigen against which they were raised. The ELISA assay results shown in Exhibit 6 were obtained using affinity purified polyclonal rabbit antibodies raised against human VEGF-2 (Vascular Endothelial Growth Factor-2). These antibodies were tested for binding against VEGF-2 target antigen in three post-translationally processed forms of human VEGF-2 (designated as dndc, C5, and C-10), as well as against human PDGF (Platelet Derived Growth Factor); human VEGF and VEGF-4 (which are paralogs of VEGF-2); and, against mouse VEGF-2 dndc protein (which is the mouse *ortholog* of human VEGF-2 dndc). Additionally, VEGF-2 antibody binding was also compared to rabbit IgG (negative control) antibody binding. In Exhibit 6, VEGF-2 antibodies and negative control antibodies were analyzed at three different concentrations over a 10,000 fold range (*i.e.*, 0.001, 0.1, and 10 μg/mL). Antibody binding was quantified using an ELISA assay wherein the measured optical density of the assay solution increases in direct proportion to the concentration of antibodies bound to antigen.

Most significantly, the assay results shown in Exhibit 6 demonstrate that, even at the highest antibody concentration tested (when antibody is bound to VEGF-2 antigens at, or near, saturation level (*i.e.*, 10 μg/mL)), the VEGF-2 antibodies did not cross-react with PDGF or the paralogous proteins VEGF and VEGF-4. This is demonstrated by the fact that the binding profiles for VEGF-2 antibodies versus non-target antigens (*i.e.*, VEGF, VEGF-4, and PDGF) are very similar to those of the negative control antibodies versus the non-target antigens. Additionally, at antibody concentrations ranging from 0.1 to 10 μg/mL the <u>anti-human</u> VEGF-2 antibodies also exhibit a similar antibody binding profile when tested against an orthologous mouse form of VEGF-2 (compare binding profiles for "VEGF-2 dndc" versus "Mouse VEGF-2 dndc" fragments). If the VEGF-2 antibodies *had* exhibited cross-reactivity toward VEGF, VEGF-4, or PDGF (*i.e.*, if VEGF-2 antibodies were not specific for VEGF-2) then the binding profiles for the VEGF-2 antibodies versus the non-target antigens would be

similar to the binding profiles for VEGF-2 antibodies versus VEGF-2 antigens (*i.e.*, with optical densities ranging from approximately 0.5 to 2.5 over antibody concentrations ranging from 0.1 to 10 μg/ml).

In sum, the VEGF-2 antibodies tested in this assay exhibit binding specificity for VEGF-2 orthologs but no significant level of cross-reactivity with non-target antigens. Thus, Exhibit 6 demonstrates a measurable and recognizable difference between antibody binding specifically to target antigens versus non-target antigens. Exhibit 6 also demonstrates that antibodies which specifically bind a target protein may also bind various proteolytically processed forms of that protein (*i.e.*, compare antibody binding to VEGF-2 forms dndc, C5, and C-10). Additionally, the results in the Exhibit 6 demonstrate that antibodies which specifically bind the protein against which they were raised may also specifically bind orthologs of that protein, but not paralogs of that protein (*compare*, VEGF-2 antibodies bound <u>mouse</u> VEGF-2 and <u>human</u> VEGF-2 (orthologs), but not human <u>VEGF</u> or human <u>VEGF-4</u> (paralogs)).

V. *Conclusion*

A. In sum, on or before March 8, 1994 an antibody that specifically bound a protein was understood to be an antibody that under empirically optimized antibody binding conditions:

a) was useful in biological assays, diagnostic assays, or therapeutic protocols because of its ability to discriminate between the target protein and non-target proteins;

b) bound the protein against which it was raised/screened with significantly higher affinity than it bound other proteins (*i.e.*, paralogues and unrelated proteins); and,

c) might also bind fragments of the protein and/or variants of the protein against which it was raised/screened (*e.g.*, post-translationally processed forms of the protein, orthologous proteins, and proteins encoded by alternative alleles or alternatively spliced transcripts).

B. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patents issuing thereupon.

21 Apr 04

DATE

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Education

1985, Ph.D. (Molecular Biology). Institute of Bioorganic Chemistry. Novosibirsk, Russia.
Thesis: Development of new methods of immunoanalysis using human myoglobin as a model protein.
1977, M.S. (Biochemistry). Novosibirsk University, Novosibirsk, Russia.

Research experience

1997-Present. Human Genome Sciences, Inc. Rockville, MD.
- 1997-1998 - Scientist, Cell biology,
- 1998-2001 - Section Head, Antibody Development
- 2001-2003 - Associate Director, Antibody Development
- 2003-Present – Director, Clinical Immunology and Antibody Development.

Currently supervise a group of 42 employees.
Responsible for antibody and ELISA development to support pre-clinical and clinical studies on novel therapeutic proteins and antibodies. Developed antibodies and ELISA assays for more than 30 proteins in early pre-clinical development. Supervise development and qualification of late stage pre-clinical and clinical PK, immunogenicity and neutralization assays for PAmAb, ETR-2, TR2-J, ETR-1, anti-CCR5 mAb, Albuferon.
Since 1999 started a program of generation and characterization of human therapeutic antibodies using Xenomouse technology. The main interest is concentrated on antibodies to TNF-family members and integral transmembrane proteins (7 TMs).
Identified and characterized several new cancer specific targets for antibody mediated immunotherapy using electronic library class analysis of the HGS database.

1992-1997. Lab. of Genetics, NCI, NIH. Bethesda, MD. Visiting Associate.
Conduct research on cellular and molecular mechanisms of development and dissemination of murine B-cell tumors. Main fields of activity are:
Studies on structural aspects of interaction of primary plasma cell tumors with stromal elements of the inflammatory granulomatous tissue (establishing primary cultures of stromal cells from mouse granuloma tissu)..
Generation of rat and rat x mouse monoclonal antibodies, screening MAB by ELISA on immobilized primary stromal cells and by immunohistochemical staining of cloned stromal cells, selection of specific clones by immunoperoxidase staining of frozen sections of different tissues.

Investigation of c-myc genomic instability by frequency determination of spontaneous and cholera toxin induced chromosomal translocations in mice susceptible and resistant to plasmacytomagenesis (single copy Long distance PCR followed by Southern blot analysis using "nested" oligonucleotide probes). Assessment of the role of Il-4 and γ-interferon in plasmacytoma development by tumor induction experiments in Il-4 and γ-interferon knockout mice (viral tumor induction, tumor phenotyping by FACS analysis, ELISA, RFLP analysis and histochemistry).

Investigation of disseminated growth of murine plasmacytoma with particular attention to the question of bone marrow involvement (construction of tagged cell lines, intravenous injection in mice, dissection of mice, preparing tissue specimens for paraffin sections and DNA isolation, detection of metastases by PCR and immunohistochemistry, assessing the bone marrow involvement and bone resorption by microscopic analysis of specially stained paraffin sections).

Generation of "knockout" mice using homologous recombination technology (preparation and characterization of targeting constructs, transfection and selection of embryonic stem cells, limited experience in blastocyst injection and reimplantation into foster mothers). Investigation of the role of different genes (CD44, Sα and Pvt-1) in plasmacytomagenesis.

1988-1992. Director, Research and Development. BioSAN Inc., Institute of Bioorganic Chemistry, Siberian Branch of the USSR Academy of Sciences, Novosibirsk.

Responsible for Research and Development of a small company affiliated with an academic institution. Supervise a group of 15 employees including 5 doctoral level biologists and chemists. Major directions include development of new immunochemical methods for research and diagnostic purposes, development of an universal approach for random tritium labeling of organic compounds and biopolymers, development of an ultrafast oligonucleotide synthesizer.

Personal research interests concentrate on development of monoclonal antibodies and new immunochemical methods for detection of low molecular weight toxicants.

1987-1989. Lab chief. Laboratory of Immunochemistry and Cellular Engineering, Institute of Bioorganic Chemistry and affiliate Institute of Clinical Immunology, Siberian Branch of the USSR Academy of Sciences, Novosibirsk.

Provided scientific and administrative leadership for 7 employees including 2 postdoctoral biochemists. The main purposes of the laboratory were facilitation of collaboration between academic and clinical institutions, developing new diagnostic tools, and establishing a training program for medical school graduates.

Conducted research on time resolved fluoroimmunometric analysis. Involved in immunochemical studies of the topography and functional mapping of RNA-polymerases from different species, using monoclonal antibodies.

1984-1987. Staff Scientist. Institute of Bioorganic Chemistry, Siberian Branch of the USSR Academy of Sciences. Novosibirsk.

Established hybridoma technology using domestic reagents and components (one of the first groups in USSR to establish hybridoma technology). Generated monoclonal antibodies against a variety of antigens including human myoglobin, tick borne encephalitis virus, E.coli and Wheat germ RNA-polymerases. Developed an original method for localization of antigenic determinants in proteins with known primary structure by means of limited protein hydrolysis and immunochemical staining of Western blots.

1979-1984. Institute of Organic Chemistry, Siberian Branch of USSR Academy of Sciences. Novosibirsk. Research Fellow, since 1981 - Junior Scientist.

Conduct research on new principles of immunoanalitical techniques. Developed a rapid method of nonequilibrium radioimmunoassay of human myoglobin for express diagnostics of myocardial infarction.

Synthesized ATP conjugates with thyroxine and myoglobin and developed a new technique of ATP-metric bioluminescent immunoassay. Proved the possibility to use complement mediated antigen-antibody specific lysis of liposomes as an immunoanalytical tool.

Developed technologies for industrial production of RIA kits for myoglobin and insulin.

1975-1979. Institute of Cytology and Genetics, Siberian Branch of USSR Academy of Sciences, Novosibirsk. Research Student, since 1977 - Research Fellow.

Research on molecular mechanisms of steroid hormones action. Demonstrated increasing of in vitro translation activity of mRNA preparations from rat liver upon hydrocortisone administration

Specific areas of expertise:

Antibody development.

Involved in Antibody development since 1979.

During the last six years supervised the generation and characterization of MABs against more than 30 proprietary HGS proteins.

Generated and characterized MABs against a number of haptens, protein, viral and cellular antigens: 2,3,7,8 - tetrachlorodibenzodioxine, myoglobin, murine G-6-PDH (allele specific), RNA polymerase from E.coli (different subunits), RNA-polymerase from wheat germs (different subunits, topographic determinants), tick born encephalitis virus (different proteins, topographic determinants), murine stromal cells (surface specific, lineage specific, tissue specific).

Generated and characterized human monoclonal antibodies against a more than 10 proprietary HGS targets using Xenomouse technology. Experience in generation of Mabs against integral transmembrane proteins.

Established an efficient strategy of generation of poly and monoclonal anti-idiotypic antibodies in rabbits and mice.

Assay development.

Supervised Development and Qualification of PK, immunogenicity and neutralization assays in a GLP compliant environment.

Established a number of assays for screening and characterization of murine and human (both phage display and Xenomouse) antibodies – affinity ranking, receptor binding, real time kinetics (BIACore), cell surface binding, complement fixation, complement and cell mediated antibody cytotoxicity, cell proliferation, cell viability.

Established a strategy for screening MAB against cell surface antigens of stromal cells (ELISA and DELFIA on immobilized cells of different lineages; high-throughput FACS analysis, immunostaining on tissue culture clusters, frozen and paraffin sections; Western blot analysis of cell extracts).

Established assays for pre-clinical and clinical PK, immunogenicity and neutralization studies for more than 20 different proteins and antibodies.

Developed a method for mapping MAB epitopes on proteins (limited enzymatic or chemical degradation of the protein of interest, separation of peptides by PAGE and subsequent Western blot immunostaining). Characterized epitopes for MAB against human myoglobin, RNA polymerases from E.coli, wheat germs, and V3 protein from tick-borne encephalitis virus).

Scientific publications.

Book chapter:

Grachev M.A., Roschke V.V. ATP-metric immunoanalysis. *In:Luminescence immunoassay and molecular applications*, p203-215, K.Van Dyke, R.Van Dyke Eds. CRC Press, Boca Raton, 1990.

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IMMUNOLOGY
A SYNTHESIS
Second Edition

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PART-OPENING ELECTRON MICROGRAPHS

Part 1 (page 19): Antibody–hapten complex (purified rabbit anti-2,4-dinitrophenyl antibody and a bivalent hapten). [From Valentine and Green, 1967. *J. Mol. Biol.* 27: 615]

Part 2 (page 191): A resting lymphocyte, probably a T cell (×21,800). [Courtesy of D. Zucker-Franklin, New York University Medical Center]

Part 3 (page 543): Immune complexes, seen as electron-dense, hump-shaped deposits in the upper third of the photo, along a capillary wall in a glomerulus following streptococcal glomerulonephritis (×17,250). [Courtesy of M. N. Yum, Indiana University Medical Center]

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free hapten. In general, carriers are molecules that are of themselves immunogenic. Hence we may think of the hapten as an added determinant on an already immunogenic molecule. The study of hapten–carrier systems has given us much information about the nature of antigens and the antigen–antibody interaction, but it has also been one of the keys to understanding the cellular events in the immune response (see Chapter 17).

The Specificity of Serological Reactions

Landsteiner studied haptens and carriers in an attempt to work out the rules that govern antigenicity. The compilation of these studies appeared in his classic treatise, *The Specificity of Serological Reactions*. As we will see, no universal rules governing antigenicity came out of this work, but what did emerge was the realization that the chemical properties of the antigen molecule determine the specificity of the immune system. SPECIFICITY is defined as the ability of antibodies produced in response to an antigen to react with that antigen and not with others. The thoroughness of Landsteiner's approach and the elegance of his thought make browsing in this volume, which is available in paperback, a worthwhile experience for any scientist.

Landsteiner immunized a rabbit with a hapten–carrier conjugate. This injection resulted in antiserum with both anti-hapten and anti-carrier activity. He then conjugated the hapten to a different carrier and reacted the conjugate with the same antiserum to test for the presence of *anti-hapten* antibodies. Because he had changed carrier molecules for the test, there was no anti-carrier reaction; the reaction observed was between the anti-hapten antibodies and the hapten. He then varied the properties of the hapten in order to study, for example, the effect of acidic or ionic groups on the ability of the antibody raised against the original hapten to react with the modified hapten. Although no general rules emerged, it is instructive to look at some of Landsteiner's conclusions (Landsteiner, 1962):

> The principal results of numerous precipitin tests with azoproteins were the following . . .
>
> 1. First of all, the nature of the acidic groups was of decisive influence. [p. 163]

Data from Landsteiner's experiments are shown in Tables 1–4. Antibody is raised against aminobenzene or aminobenzene with

Cross Reactivity

Antibody molecules can exhibit great specificity, but there are CROSS REACTIONS—cases in which antibody to antigen A also reacts with antigen B. This can be due to the presence of the same molecular configuration, or ANTIGENIC DETERMINANT, on the two antigens, or to properties of a determinant that allow it to be recognized as though it were another group. Antigenic determinants are also called *epitopes*. As we move through the book we will use these terms almost interchangeably. We can conceive of molecules that have similar but not identical structures and appear in closely related species. These molecules may have enough similarity to allow antibodies against one to react with the other.[1]

Table 5 shows the percentage of cross reactivity between albumins of different species. Antibody was made against bovine serum albumin (BSA), and the extent of the ability of albumins from other species to react with the anti-BSA was then determined. This cross reactivity is probably due to the presence of common determinants on the different albumins. To determine this, however, each of the determinants must be isolated and studied chemically. Even then, as we will see later in this chapter, we cannot be quite certain of

[1] The neurobiologist A. K. Hall has suggested the term IMMUNOFREQUENT for such determinants.

Table 5 Cross reaction between BSA and other albumins.[a]

Albumin source	Percentage of cross reactivity with BSA	Albumin source	Percentage of cross reactivity with BSA
Human	15	Mouse	10
Pig	32	Rat	13
Sheep	[illegible]	Hamster	13
Horse	13	Cat	25
Guinea pig	[illegible]	Vallaroo	[illegible]
Dog	13		

Source: Data from Weigle. 1961. *J. Immunol.* 87: 599.

[a] Rabbit anti-BSA was absorbed with each of the albumins listed and then tested for its ability to react with BSA. This ability is expressed as a percentage *cross reactivity*. The data show that sheep BSA has the highest amount of cross reactivity and guinea pig and vallaroo the least.

O

Top

Orthologue

Orthologues are genes derived from a common ancestor through vertical descent. This is often stated as the same gene in different species. In contrast, paralogs are genes within the same genome that have evolved by duplication.

The hemoglobin genes are a good example. Two separate genes (proteins) make up the molecule hemoglobin (alpha and beta). The alpha and beta DNA sequences are very similar and it is believed that they arose from duplication of a single gene, followed by separate evolution in each of the sequences. Alpha and beta are considered paralogs. Alpha hemoglobins in different species are considered orthologs.

P

Top

Paralog

Paralogs are usually described as genes within the same genome that have evolved by duplication. See Ortholog.

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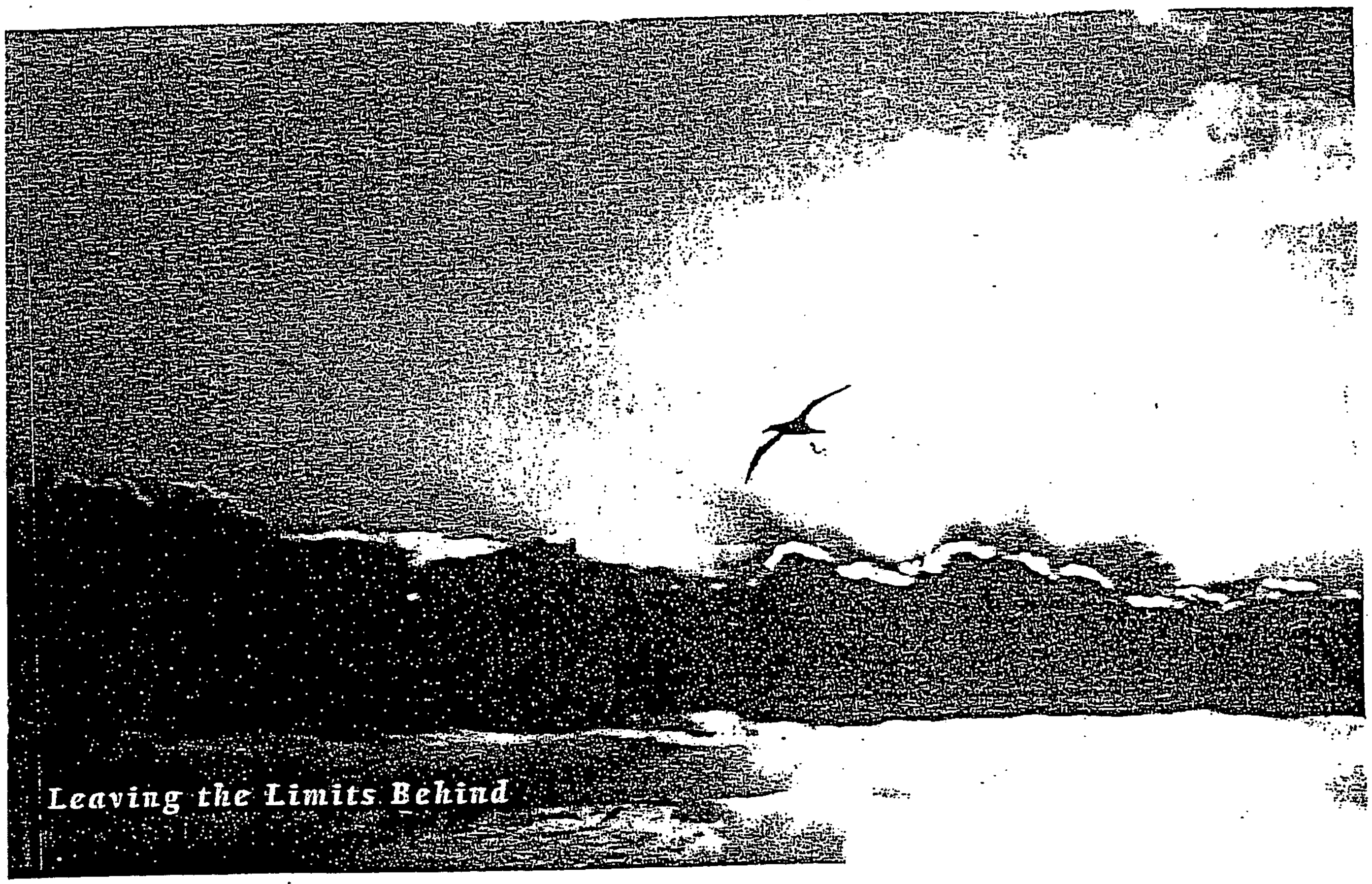

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CD	Antibody	Specificity and Notes	Species Specificity
	Antibodies to Human Leukocyte Antigens		
CD1a	**Anti-thymocyte** (clone VIT6b) Cat. No. 1273 418	The antibody recognizes the heavy chain (49 kD) of the CD1a molecule and binds thymocytes, Langerhans cells in human skin, astrocytes in human brain, and endothelial cells in human spleen. The antibody may be used to detect thymocytes in suspensions of blood cells. **Stability:** Lyophilizate is stable at +4°C. Reconstituted antibody solution (200 µg/ml in water) is stable at −20°C. Avoid repeated freezing and thawing.	human
CD2	**Anti-T pan** (clone MT26) Cat. No. 1089 242	The antibody recognizes the CD2 antigen (46-50 kD) on most peripheral T lymphocytes (>70% of resting T cells and >80% of activated T cells) and thymocytes (80%) in humans. The antibody also binds T cells in frozen tissue sections of lymph and thymus. The CD2 antigen is the sheep erythrocyte receptor found on most human T lymphocytes. **Stability:** Lyophilizate is stable at +4°C. Reconstituted antibody solution (200 µg/ml in water) is stable at −20°C. Avoid repeated freezing and thawing.	human
CD3	**Anti-T pan** (clone 4B5) Cat. No. 1273 485	The antibody recognizes the CD3 complex (three proteins, 19–29 kD) on all peripheral T lymphocytes and mature thymocytes in humans. The antibody may be used to detect the total population of mature T lymphocytes or to study the mechanism of the CD3 complex. **Stability:** Lyophilizate is stable at +4°C. Reconstituted antibody solution (200 µg/ml in water) is stable at −20°C. Avoid repeated freezing and thawing.	human
CD9	**Anti-leukemia associated antigen** (p24) (clone BA-2) Cat. No. 1118 021	The antibody reacts with a monomeric 24 kD protein found on the surface of cells from about 75% of the cases of non-T, non-B acute lymphoblastic leukemia, and on normal immature lymphoid cells (lymphoid progenitor cells, which lack characteristics of either T or B cells); reacts weakly to cells from some T-acute lymphoblastic leukemias; binds 3% of peripheral blood lymphoid cells, and about 5-10% of bone-marrow mononuclear cells. BA-2 fixes rabbit complement; binds protein A. **Stability:** Stable at +4°C; for prolonged storage, aliquot and store at −20°C or colder.	human
CD10	**Anti-common acute lymphoblastic leukemia antigen** (CALLA) (clone 12A1) Cat. No. 1295 772	The antibody recognizes CALLA, a neutral endopeptidase (90 kD) found in cells from human pre-B cells, fibroblasts, granulocytes and most acute lymphoblastic leukemias (ALL). **Stability:** Lyophilizate is stable at +4°C. Reconstituted antibody solution (200 µg/ml in water) is stable at −20°C. Avoid repeated freezing and thawing.	human
CD11a	**Anti-lymphocyte function antigen** (LFA) (clone 38) Cat. No. 1428 535	The antibody specifically recognizes the α-chain (CD11a) of the LFA-1 complex and immunoprecipitates a protein with a molecular weight of 175-180 kD from human monocyte lysates. The antibody reacts with nearly all peripheral blood leukocytes. **Stability:** Stable at +4°C; for prolonged storage, aliquot and store at −20°C or colder. Avoid repeated freezing and thawing.	human
NEW CD11b	**Anti-macrophage** (clone 44) Cat. No. 1441 256	The antibody recognizes the complement receptor type 3 (CD11b), a plasma membrane glycoprotein on human monocytes. The antibody inhibits the aggregation of activated neutrophils by greater than 50%. The antibody immunoprecipitates a protein from the surface of neutrophils with an apparent molecular weight of 165 kD. **Stability:** Stable at +4°C; for prolonged storage, aliquot and store at −20°C or colder. Avoid repeated freezing and thawing.	human
CD11b/ CD18	**Anti-Mac-1** (macrophage associated antigen) (clone M1/70) Cat. No. 1118 129	The antibody specifically reacts with native mouse and human Mac-1 (complement receptor type 3; Ly-40) antigen and precipitates two chains, 170 kD (CD11b) and 95 kD (CD18). **Stability:** Stable at −20°C. Avoid repeated freezing and thawing.	human, mouse
NEW CD11c	**Anti-lymphocyte function antigen-1α** (LFA-1α) (clone BL-4H4) Cat. No. 1484 311	The antibody specifically recognizes the α-chain (CD11a) of the LFA-1 complex and immunoprecipitates a protein with a molecular weight of 150 kD from human monocyte lysates. The antibody reacts with nearly all peripheral blood leukocytes. **Stability:** Stable for 6 months at +4°C. Alternatively, store in aliquots at −20°C. Avoid repeated freezing and thawing.	human
CD14	**Anti-myeloid cells** (clone AML2-23) Cat. No. 1118 188	The antibody is cytotoxic in the presence of complement. The antibody binds an antigen (50 kD) found on acute myeloid leukemias and some acute promyelocytic leukemias. It also binds normal monocytes, macrophages and neutrophils. **Reference:** Ball, E.D. *et al.* (1982) *Proc. Natl. Acad. Sci. USA* 79:5374. **Stability:** Stable at +4°C; for prolonged storage, aliquot and store at −20°C or colder. Avoid repeated freezing and thawing.	human
CD15	**Anti-granulocyte** (clone 4D1) Cat. No. 1295 845	The antibody recognizes lacto-N-fucopentose III, a carbohydrate antigen found on human neutrophil granulocytes. The antigen is also found on Reed-Sternberg and Hodgkin's (lymphoma) cells. The antibody also binds some secretory gland tissue and epithelium from esophagus and cervix. The antibody reacts with the CD15 antigen (expressed late in the development of granulocytes) on cells in suspension and in frozen tissue. The antibody may be used to detect mature granulocytes in suspensions of blood cells. **Stability:** Lyophilizate is stable at +4°C. Reconstituted antibody solution (200 µg/ml in water) is stable at −20°C. Avoid repeated freezing and thawing.	human

B = Southern/northern/dot blots
FC = Flow cytometry
N = Neutralizing
C = Cryosections
IC = Immunocytochemistry
P = Paraffin sections
CD = Cluster of Differentiation
IHC = Immunohistochemistry
S = Histological sections
E = ELISA
IP = Immunoprecipitation
W = Western (proteins)

Note: Listed applications are tested by Boehringer Mannheim. Any application not listed does not necessarily indicate that the antibody does not work in that application.

Ig Class	Application	Working Conc. (µg/ml)	No. of Tests	Form	Cat. No.	Pack Size	Price ($)
Mouse IgG_1 (monoclonal)	C, IC	1–5 (S, IC)	2,000–20,000 (S). 800–4,000 (FC)	Lyophilized	1273 418 1273 426	200 µg □	238.00
Mouse IgG_1 (monoclonal)	C, IC	0.4–1 (IC)	800 (FC)	Lyophilized	1089 242 1091 778	200 µg □	228.00
Mouse IgG_1 (monoclonal)	C, FC, IC	2–5 (FC)	800–10,000 (FC)	Lyophilized	1273 485 1273 493	200 µg □	238.00
Mouse IgG_3 (monoclonal)	FC	10 (FC)	200 (FC)	Solution, 500 µl	1118 021 1118 030	200 µg □	250.00
Mouse IgM (monoclonal)	C, IC	1–10 (IC)	400–4,000 (FC)	Lyophilized	1295 772 1296 965	200 µg □	238.00
Mouse IgG_{2a} (monoclonal)	C, FC, IC, IP	1–5 (FC, IC)	200–1,000 (FC,IC)	Solution, 500 µl	1428 535 1420 526	100 µg □	201.00
Mouse IgG_1 (monoclonal)	C, FC, IC, IP	2–5 (FC, IC)	100–1,000 (FC, IC)	Solution, 500 µl	1441 256 1441 221	100 µg □	237.00
Rat IgG_{2b} (monoclonal)	C, FC, IC, IP	10 (FC)	100 (FC)	Solution, 500 µl	1118 129 1118 137	100 µg □	252.00
Fluorescein conjugate	C, FC, IC	10 (FC)	100 (FC)	Solution, 500 µl	1289 942 1289 969	100 µg □	298.00
Phycoerythrin conjugate	FC, IC	10 (FC)	100 (FC)	Solution, 500 µl	1353 691 1353 705	100 µg □	358.00
Mouse IgG_1 (monoclonal)	FC, IC, IP	1–5 (FC,IC)	100–1,000 (FC, IC)	Solution, 500 µl	1484 311 1460 455	100 µg □	196.00
Mouse IgG_{2b} (monoclonal)	FC	1 µg/10^6 cells (in 100 µl) (FC)	200	Solution, 500 µl	1118 188	200 µg	238.00
Mouse IgM (monoclonal)	C, IC	1–10 (IC)	400–4,000	Lyophilized	1295 845 1296 949	200 µg □	238.00

□ Indicates special bulk quantity availability, see page ii for details

A pack insert for this product is available upon request.

Antibody	Specificity and Notes	Species Specificit
Antibodies for Neuroscience and Signal Transduction Research (continued)		
Anti-Alzheimer precursor protein A4, formalin grade (Pre-A4) (clone 22C11) Cat. No. 1285 262	The antibody was produced by immunization with the fusion protein pre-$A4_{695}$, and reacts with the human Alzheimer precursor protein A4. It shows cross-reactivity with the pre-A4 molecules from fish, rat, mouse, and monkey. **Stability:** Lyophilizate is stable at +4°C. Reconstituted antibody solution (200 µg/ml in water) is stable at −20°C. Avoid repeated freezing and thawing.	human, mc fish, monk
Anti-β-Amyloid, Alzheimer Cat. No. 1381 431	The antibody reacts with plaques in brain cryosections of Alzheimer patients. **Stability:** Lyophilizate is stable at +4°C. Reconstituted antibody solution (200 µg/ml in water) is stable at −20°C. Avoid repeated freezing and thawing.	human
NEW **Anti-Ca^{2+}/calmodulin-dependent protein kinase type II** (clone 6G9) Cat. No. 1481 703	Recognizes the α-subunit of Ca^{2+}/calmodulin-dependent protein kinase II and reacts with tissue from all mammalian species tested, as well as with chicken and frog tissues. **Stability:** Stable at −20°C.	mammals, frog
Anti-calcitonin gene-related peptide, human Cat. No. 1295 241	To obtain the polyclonal antiserum, rabbits were immunized with calcitonin gene-related peptide. The antibody is suitable for detection of CGRP on brain sections. **Stability:** Lyophilizate is stable at +4°C. Reconstituted antibody solution (200 µg/ml in water) is stable at −20°C. Avoid repeated freezing and thawing.	human, mc chicken
Anti-L-CAM/Uvomorulin (clone 6F9) Cat. No. 1441 892	The antibody specifically recognizes the 120 kD and the 80 kD band of L-CAM/Uvomorulin (Arc-1 E-cadherin cell-CAM 120/80) in man and rabbit. L-CAM/Uvomorulin staining is confined to the lateral border of epithelial cells and, within the intestine, shows more intense concentrations in the area of the junctional complex. As a positive control, the cell line MCF-7 can be used. **Stability:** Lyophilizate is stable at +4°C. Reconstituted antibody solution (200 µg/ml in water). is stable at −20°C. Avoid repeated freezing and thawing.	human, rab
Anti-choline acetyltransferase (ChAT) (clone 11-255) Cat. No. 770 981	The antibody reacts with choline acetyltransferase from monkey, pig, rat, and mouse. **Stability:** Lyophilizate is stable at −20°C. Store reconstituted antibody solution at +4°C. **Do not freeze.**	mouse, rat, monkey
Anti-choline acetyltransferase, human (ChAT) (clone 1.B3.9B3) Cat. No. 1464 272	The antibody reacts with choline acetyltransferase from man, rat, and pig. The antibody can be used for investigating the decrease of the cholinergic system in Alzheimer's disease. **Stability:** Stable at +4°C.	human, rat
Anti-chromogranin A (clone LK2H10) Cat. No. 1199 021	The antibody recognizes the 68 kD protein, chromogranin A, found exclusively in secretory storage granules of almost all neuroendocrine cells. The antibody binds small cell carcinoma of the lung, Merkel cell carcinomas and neuroblastomas weakly; binds most other endocrine-derived tumors strongly. Primary antibody for immunohistochemical detection and characterization of normal endocrine cells and endocrine-derived tumor cells [Lloyd, R.V. and Wilson, B.S. (1983) *Science* 222, 628-630; Wilson, B.S. and Lloyd, R.V. (1984) *Am. J. Pathol.* 115, 458-468]. **Stability:** Stable at +4°C for prolonged storage, aliquot and store at −20°C or colder. Avoid repeated freezing and thawing.	human, mc pig
Anti-CNP (2',3'-cyclic nucleotide 3'-phosphodiesterase) (clone 11-5B) Cat. No. 1442 007	The antibody reacts with both CNPase 1 and CNPase 2 (2',3'-cyclic nucleotide 3'-phosphodiesterase) and is used as an oligodendrocyte and Schwann cell marker. **Stability:** Lyophilizate is stable at +4°C. Reconstituted antibody solution (200 µg/ml in water) is stable at −20°C. Avoid repeated freezing and thawing.	human, mc rabbit, bovi sheep
NEW **Anti-dystrophin** (clone 1808C) Cat. No. 1558 722	The antibody recognizes dystrophin, the 427 kD product of a 14 kb transcript encoded by the Duchenne muscular dystrophy gene locus on chromosome Xp21. It does not label human Duchenne muscle or mouse mdx (murine muscular dystrophy) tissue, and it does not cross-react with proteins closely related to dystrophin (*e.g.*, C-protein, α-actin, or human muscle spectrin). **Reference:** Hoffman, E.P., Brown, R.H. and Kunkel, L.M. (1987) *Cell* 51,919. **Stability:** The antibody is stable for 18 months at +4°C or 2 years at −20°C. Once reconstituted, store the antibody in aliquots at −20°C to avoid repeated freezing and thawing. Avoid gross bacterial contamination.	human, rat other mam chicken, fr
Anti-β-Endorphin (clone 3-E7) Cat. No. 1089 170	The antibody reacts with the NH2-terminal Tyr-Gly-Gly-Phe-sequence of human β-endorphin. Therefore, there is a high cross-reactivity with homologues with the same sequence like (Met)-enkephalin and (Leu)-enkenhalin and many opioid peptides. The antibody reacts with β-endorphin from pig and camel. **Stability:** Lyophilizate is stable at −20°C. Store the reconstituted antibody solution at +4°C.	human, pig
Anti-$GABA_A$ receptor, α-chain (Anti-γ-aminobutyric acid) (clone bd 24) Cat. No. 1381 440	The antibody reacts with the α-chain of $GABA_A$ receptor from cow and rat. **Stability:** Lyophilizate is stable at +4°C. Reconstituted antibody solution (200 µg/ml in water) is stable at −20°C. Avoid repeated freezing and thawing.	human, bo
Anti-$GABA_A$ receptor, β-chain (Anti-γ-aminobutyric acid) (clone bd 17) Cat. No. 1381 458	The antibody reacts with the β-chain of $GABA_A$ receptor from cow, man, and rat. **Stability:** Lyophilizate is stable at +4°C. Reconstituted antibody solution (200 µg/ml in water) is stable at −20°C. Avoid repeated freezing and thawing.	human, rat cat

B = Southern/northern/dot blots · C = Cryosections · CD = Cluster of Differentiation · E = ELISA
FC = Flow cytometry · IC = Immunocytochemistry · IHC = Immunohistochemistry · IP = Immunoprecipit
N = Neutralizing · P = Paraffin sections · S = Histological sections · W = Western (protei

Note: Listed applications are tested by Boehringer Mannheim. Any application not listed does not necessarily indicate that the antibody does not work in that application.

Ig Class	Application	Working Conc. (µg/ml)	No. of Tests	Form	Cat. No.	Pack Size		Price ($)
Mouse IgG_1 (monoclonal)	C, P, W	5–10	250–1,000 (S)	Lyophilized	1285 262 1272 829	50 µg ☐	🗎	252.00
Rabbit Ig (polyclonal)	C, P, W	10–20 (S), 20 (W)	50–500 (S)	Lyophilized	1381 431 1384 082	100 µg ☐	🗎	244.00
Mouse IgG_1 (monoclonal)	C, W	10 (IC, W)	200 (IC)	Lyophilized	1481 703 1484 711	200 µg ☐	🗎	237.00
Rabbit IgG (polyclonal)	C, P	5–10	250–500 (S)	Lyophilized	1295 241 1284 576	50 µg ☐	🗎	202.00
Mouse IgG_1 (monoclonal)	C, W, IC, E	1–4 (IC)	250–500 (IC)	Lyophilized	1441 892	50 µg	🗎	Inquire
Rat IgG (monoclonal)	C, P (limited application)	2.5–4 (S)	125–200 (S) 500–800 (S)	Lyophilized Lyophilized	770 981 770 990 755 702	10 µg 40 µg ☐	🗎	116.00 329.00
Mouse IgG_1 (monoclonal)	C, W P (limited application)	10–20 (CS)	100–200 (S)	Lyophilized Lyophilized	1464 272 1372 432 1372 025	50 µg 100 µg ☐	🗎	180.00 438.00
Mouse IgG_1 (monoclonal)	C, P	1–10 (S)	2,500–25,000 (S)	Solution, 500 µl	1199 021 1199 030	500 µg ☐	🗎	330.00
Mouse IgG_1 (monoclonal)	C, W, IC	10 (IC)	100–200 (IC)	Lyophilized	1442 007 1442 015	100 µg	🗎	195.00
Mouse IgG_1 (monoclonal)	IHC				1558 722 1558 749	100 µg ☐	🗎	155.00
Mouse IgG_{2a} (monoclonal)	E	1 (E)	400 (E)	Lyophilized	1089 170 1091 395	40 µg ☐	🗎	216.00
Mouse IgG_1 (monoclonal)	C, W, IP P (limited application)	10–20 (S), 20 (W)	100–200 (S)	Lyophilized	1381 440 1381 261	100 µg ☐	🗎	249.00
Mouse IgG_1 (monoclonal)	C, W, IP P (limited application)	10–20 (C), 20 (W)	50–500 (C)	Lyophilized	1381 458 1381 288	100 µg ☐	🗎	249.00

☐ Indicates special bulk quantity availability, see page ii for details.

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ASSAYS FOR ANTIBODY PRODUCTION

All assays for antibody production depend upon the measurement of the interaction of elicited antibody with antigen. This binding of antigen by antibody can be measured in solution by methods such as equilibrium dialysis or fluorescence quenching, by measuring the production of insoluble antigen-antibody complexes in aqueous solutions or gels, or by measuring the adsorption of soluble antigen to solid-phase antibody (or conversely, the binding of soluble antibody to solid-phase antigen). It is far beyond the scope of this manual, and unnecessary in any but a historical context, to consider even a large sampling of the available methods for measuring antibodies or antigens. For modern practical purposes, most investigators require methods that are rapid, sensitive, specific, quantitative (at least in terms of comparison to some known standard), and reproducible. Ideally, the methods should not require particularly expensive reagents or equipment, and they should be effective for measuring both polyclonal antibodies as well as monoclonal antibodies. These methods should also be useful in evaluating antigens when known amounts of specific antibodies are available.

This section describes two general methods for assaying antibodies and antigens, representative of both extremes of chronological development—the enzyme-linked immunosorbent assay (ELISA; UNITS 2.1 & 2.2), a highly versatile, sensitive, and quantitative technique that requires little equipment and for which critical reagents are readily available, and the double-immunodiffusion assay (UNIT 2.3), which requires nothing more than a glass slide, Pasteur pipet, agar, and phosphate-buffered saline. Both assays are useful for evaluating antibody activity of polyclonal antisera, but by and large, the double-immunodiffusion assay is not useful for evaluating binding of monoclonal antibodies to monovalent antigens. The versatility of ELISAs is emphasized by the six distinct ELISA protocols presented in UNIT 2.1: an indirect ELISA to detect specific antibodies, a direct competitive ELISA to detect soluble antigens, an antibody-sandwich ELISA to detect soluble antigens, a double antibody-sandwich ELISA to detect specific antibodies, and two cellular ELISAs, one to detect cell-surface antigens, and one to detect antibodies specific for surface antigens. The advantages of each of these assays for particular applications are summarized in Table 2.1.1. Many additional variations exist, and the specific needs of a particular problem may be better served by minor changes and adaptations of these protocols; a particular adaptation of the antibody-sandwich ELISA for isotyping antibodies is presented in UNIT 2.2.

Other methods described elsewhere in this manual may also be effectively employed as assays for antibodies or antigens. Direct or indirect immunofluorescence (using cytofluorimetry) of appropriately selected cell lines or tissues can be used to obtain both qualitative and quantitative information about the binding of an antibody or antiserum (UNITS 5.1-5.4). In addition, antibodies specifically required for either immunoprecipitation (UNIT 8.3) or immunoblotting (UNIT 8.10), including antipeptide antibodies, may be best assayed by these same methods. However, as long as a carefully designed assay is chosen for a particular antibody or antiserum, the ultimate result will be not only a high-quality reagent, but a quantitative and sensitive means of identifying the antigen in question. The ability to identify new antigens with carefully raised antibodies is limited only by the investigator's imagination and diligence.

Enzyme-Linked Immunosorbent Assays

This unit describes six different ELISA systems for the detection of specific antibodies, soluble antigens, or cell-surface antigens. In all six systems, soluble reactants are removed from solution after specifically binding to solid-phase reactants. Table 2.1.1 summarizes the different ELISA protocols, which are illustrated in Figures 2.1.1-2.1.6.

In the first four protocols, solid-phase reactants are prepared by adsorbing an antigen or antibody onto plastic microtiter plates; in the next two protocols, the solid-phase reactants are cell-associated molecules. In all protocols, the solid-phase reagents are incubated with secondary or tertiary reactants covalently coupled to an enzyme. Unbound conjugates are washed out and a chromogenic or fluorogenic substrate is added.

Table 2.1.1 Summary of ELISA Protocols

ELISA protocol	Uses	Required reagents	Comments
Indirect	Antibody screening; epitope mapping	Antigen, pure or semipure: test solution containing antibody; enzyme conjugate that binds Ig of immunized species	Does not require the use of preexisting specific antibodies; requires relatively large amounts of antigen
Direct competitive	Antigen screening; detect soluble antigen	Antigen, pure or semipure; test solution containing antigen; enzyme-antibody conjugate specific for antigen	Rapid assay with only two steps; excellent for measuring antigenic cross-reactivity
Antibody-sandwich	Antigen screening; detect soluble antigen	Capture antibody (purified or semi-purified specific antibody); test solution containing antigen; enzyme-antibody conjugate specific for antigen	Most sensitive antigen assay; requires relatively large amounts of pure or semi-pure specific antibody (capture antibody)
Double antibody–sandwich	Antibody-screening; epitope mapping	Capture antibody: (specific for Ig of immunized species); test solution containing antigen; enzyme-antibody conjugate specific for antigen	Does not require purified antigen; relatively long assay with five steps
Direct cellular	Screen cells for expression of antigen; measure cellular antigen expression	Cells that express antigen of interest; enzyme-antibody conjugate specific for cellular antigen	Sensitive assay for bulk screening; insensitive to heterogeneity of expression in mixed population of cells
Indirect cellular	Screen for antibodies against cellular antigens	Cells used for immunizing; test solution containing antibodies; enzyme conjugate that binds Ig of immunized species	May not detect antibodies specific for cellular antigens expressed at a low density

As the substrate is hydrolyzed by the bound enzyme conjugate, a colored or fluorescent product is generated. Finally, the product is detected visually or with a microtiter plate reader. The amount of product generated is proportional to the amount of analysate in the test mixture. The first support protocol can be used to optimize the different ELISAs. The second support protocol presents a method for preparing alkaline phosphatase conjugates.

BASIC PROTOCOL

INDIRECT ELISA TO DETECT SPECIFIC ANTIBODIES

This assay is useful for screening antisera or hybridoma supernatants for specific antibodies when milligram quantities of purified or semi-purified antigen are available (1 mg of purified antigen will permit screening of 80 to 800 microtiter plates; Fig. 2.1.1). Antibodies are detected by coating the wells of microtiter plates with antigen, incubating the coated plates with test solutions containing specific antibodies, and washing away unbound antibodies. A solution containing a developing reagent, (e.g., alkaline phosphatase conjugated to protein A, protein G, or antibodies against the test solution antibodies) is then added to the plate. After incubation, unbound conjugate is washed away and substrate solution is added. After a second incubation, the amount of substrate hydrolyzed is assessed with a spectrophotometer or spectrofluorometer. The measured amount is proportional to the amount of specific antibody in the test solution. Visual inspection can also be used to detect hydrolysis.

Materials

Developing reagent: protein A–alkaline phosphatase conjugate (Sigma #P9650), protein G–alkaline phosphatase conjugate (Calbiochem #539304), *or* anti-Ig-alkaline phosphatase conjugate (second support protocol)
Antigen solution
PBS (APPENDIX 2) containing 0.05% NaN_3 (PBSN)
Water, deionized or distilled
Blocking buffer
Test antibody samples
4-methylumbelliferyl phosphate (MUP) *or* *p*-nitrophenyl phosphate (NPP) substrate solution
0.5 M NaOH (optional)

Multichannel pipet and disposable pipet tips
Immulon 2 (Dynatech #011-010-3450), Immulon 4 (Dynatech #011-010-3850), or equivalent microtiter plates
Plastic squirt bottles
Microtiter plate reader (optional)—spectrophotometer with 405-nm filter or spectrofluorometer (Dynatech #011-970-1900) with 365-nm excitation filter and 450-nm emission filter

Determine developing reagent and antigen concentrations

1. Determine the optimal concentration of the developing reagent (conjugate) by criss-cross serial-dilution analysis (see first support protocol).

   Good conjugates of many specificities are available commercially. Choice of developing reagent (i.e., conjugate) is determined by the goals of the assay. If it is necessary to detect all antibodies that bind to antigen, conjugates prepared with antibodies specific for Ig κ and λ light chains should be used. Alternatively, protein A– or protein G–enzyme conjugates may be preferable when screening monoclonal antibodies. Specific monoclonal antibodies that bind protein A or protein G are easy to purify and characterize.

2. Determine the final concentration of antigen coating reagent by criss-cross serial-dilution analysis (see first support protocol). Prepare an antigen solution in PBSN at this final concentration. The final concentration of antigen is usually 0.2 to 10.0 µg/ml. Prepare ~6 ml antigen solution for each plate.

   Pure antigen solution concentrations are usually ≤2 µg/ml. Although pure antigen preparations are not essential, >3% of the protein in the antigen solution should be the antigen. The total concentration of protein in the antigen solution should be increased for semipurified antigen preparations. Do not raise the total protein concentration in the antigen solution to >10 µg/ml, since this concentration usually saturates >85% of the available sites on Immulon microtiter plates. For some antigens, coating may occur more efficiently at different pHs.

Coat plate with antigen

3. Using a multichannel pipet and tips, dispense 50 µl antigen solution into each well of an Immulon microtiter plate. Tap or shake the plate to ensure that the antigen solution is evenly distributed over the bottom of each well.

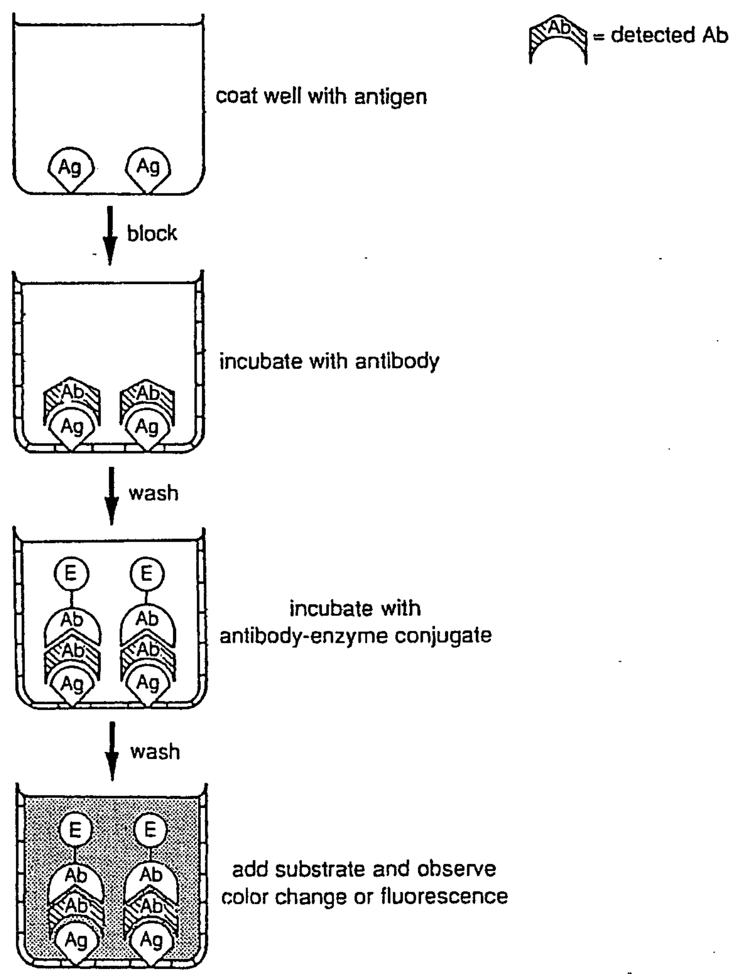

Figure 2.1.1 Indirect ELISA to detect specific antibodies. Ag = antigen; Ab = antibody; E = enzyme.

4. Wrap coated plates in plastic wrap to seal and incubate overnight at room temperature or 2 hr at 37°C.

   Individual adhesive plate sealers are sold commercially but plastic wrap is easier to use and works as well. Sealed plates can be stored at 4°C with antigen solution for months.

5. Rinse coated plate over a sink by filling wells with deionized or distilled water dispensed either from a plastic squirt bottle or from the tap. Flick the water into the sink and rinse with water two more times, flicking the water into the sink after each rinse.

Block residual binding capacity of plate

6. Fill each well with blocking buffer dispensed as a stream from a squirt bottle and incubate 30 min at room temperature.

   Residual binding capacity of the plate is blocked in this step. Tween 20 (0.05%) by itself is more effective at blocking than any protein tested, but because the combination of protein and Tween 20 may be more effective than Tween 20 alone in some cases, bovine serum albumin (BSA; 0.25%) is included in the blocking buffer.

7. Rinse plate three times in water as in step 5. After the last rinse, remove residual liquid by wrapping each plate in a large paper tissue and gently flicking it face down onto several paper towels laying on the benchtop.

   Rinsing with water is cheaper and easier than rinsing with buffered solutions and is as effective.

Add antibody to plate

8. Add 50 µl antibody samples diluted in blocking buffer to each of the coated wells, wrap plate in plastic wrap, and incubate ≥2 hr at room temperature.

   While enough antibody may be bound after 1 to 2 hr to generate a strong signal, equilibrium binding is generally achieved after 5 to 10 hr. Thus, the specific signal may be significantly increased by longer incubations.

   For this and all steps involving the delivery of aliquots of many different solutions to microtiter plates with multichannel pipets, such as the primary screening of hybridoma supernatants, the same pipet tips can be reused for hundreds of separate transfers. Wash tips between transfers by expelling any liquid remaining in the tips onto an absorbent surface of paper tissues, rinsing tips five times in blocking buffer, and carefully expelling any residual liquid from tips onto the tissues. Avoid bubbles in the tips; any tip with intractable bubbles should be replaced.

Wash the plate

9. Rinse plate three times in water as in step 5.

10. Fill each well with blocking buffer, vortex, and incubate 10 min at room temperature.

    Plates are vortexed to remove any reagent remaining in the corners of the wells.

11. Rinse three times in water as in step 5. After the final rinse, remove residual liquid as in step 7.

Add developing reagent to plate

12. Add 50 µl developing reagent in blocking buffer (at optimal concentration determined in step 1) to each well, wrap in plastic wrap, and incubate ≥2 hr at room temperature.

    The strength of the signal may be increased by longer incubations (see annotation to step 8).

13. Wash plates as in steps 9 to 11.

After final rinsing, plates may be wrapped in plastic wrap and stored for months at 4°C prior to adding substrate.

Add substrate and measure hydrolysis

14. Add 75 µl MUP or NPP substrate solution to each well and incubate 1 hr at room temperature.

15. Monitor hydrolysis qualitatively by visual inspection or quantitatively with a microtiter plate reader (see below). Hydrolysis can be stopped by adding 25 µl of 0.5 M NaOH.

    a. Visually, hydrolysis of NPP can be detected by the appearance of a yellow color. If using a microtiter plate reader to measure NPP hydrolysis, use a 405-nm filter.

    b. Visually, hydrolysis of MUP can be monitored in a darkened room by illumination with a long-wavelength UV lamp. If using a microtiter plate spectrofluorometer to measure MUP hydrolysis, use a 365-nm excitation filter and a 450-nm emission filter.

    The fluorogenic system using the MUP substrate is 10 to 100 times faster than the chromogenic system using NPP. Furthermore, the rate of spontaneous hydrolysis of MUP is much lower than that of NPP.

    To detect bound antibodies that are present at low concentration, measure hydrolysis at a later time. To calculate when to measure hydrolysis the second time, remember that the amount of hydrolysis is almost directly proportional to the time of hydrolysis. For example, if the hydrolysis in the wells of interest reads 200 at 1 hr and a reading of 2000 is desired, incubate the plate ~10 hr before taking the second reading.

ALTERNATE PROTOCOL

DIRECT COMPETITIVE ELISA TO DETECT SOLUBLE ANTIGENS

This assay is used to detect or quantitate soluble antigens and is most useful when both a specific antibody and milligram quantities of purified or semi-purified antigen are available (Fig. 2.1.2). To detect soluble antigens, plates are coated with antigen and the binding of specific antibody-enzyme conjugates to antigen-coated plates is inhibited by test solutions containing soluble antigen. After incubation with mixtures of the conjugate and inhibitor in antigen-coated wells, unbound conjugate is washed away and substrate is added. The amount of antigen in the test solutions is proportional to the inhibition of substrate hydrolysis and can be quantitated by interpolation onto an inhibition curve generated with serial dilutions of a standard antigen solution.

The direct assay may also be adapted as an indirect assay by substituting specific antibody for specific antibody-enzyme conjugate. The amount of specific antibody bound is then detected using a species-specific or isotype-specific conjugate as a tertiary reactant.

Additional Materials

Specific antibody–alkaline phosphatase conjugate (second support protocol)
Standard antigen solution
Test antigen solutions
Round- or cone-bottom microtiter plates

1. Determine the optimal concentration of coating reagent and antibody–alkaline phosphatase conjugate by criss-cross serial-dilution analysis in which the concentrations of both the antigen (coating reagent) and the conjugate (developing reagent) are

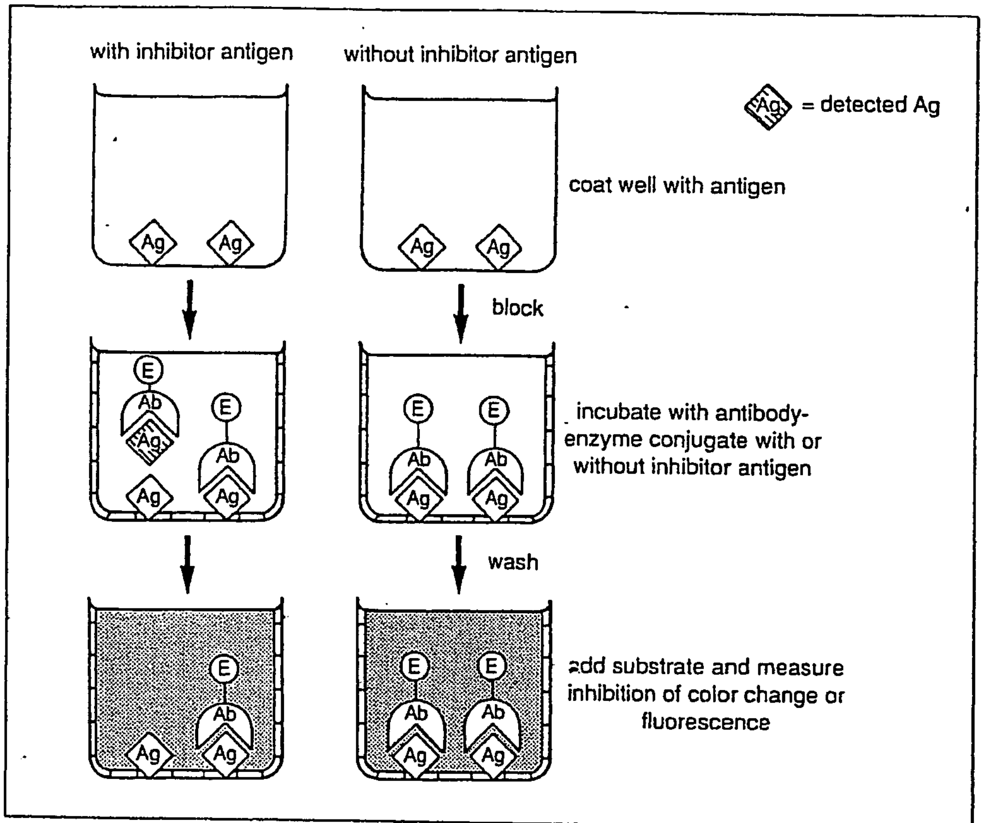

Figure 2.1.2 Direct competitive ELISA to detect soluble antigens. Ag = antigen; Ab = antibody; E = enzyme.

varied (see first support protocol). Prepare a 2× conjugate solution by diluting the specific antibody–alkaline phosphatase conjugate in blocking buffer to twice the optimal concentration.

The final concentration is usually 25 to 500 ng antibody/ml. Prepare 3 ml antibody–alkaline phosphatase conjugate for each plate.

2. Coat and block wells of an Immulon microtiter plate with 50 µl antigen solution as in steps 2 to 7 of the basic protocol.

3. Prepare six 1:3 serial dilutions of standard antigen solution in blocking buffer (see first support protocol for preparation of serial dilutions)—these antigen concentrations will be used in preparing a standard inhibition curve (see step 10).

Antigen concentrations should span the dynamic range of inhibition. The dynamic range of inhibition is defined as that range of inhibitor concentrations wherein changes of inhibitor concentration produce detectable changes in the amount of inhibition. The dynamic range of inhibition is empirically determined in an initial assay in which antigen concentration is typically varied from the micromolar (10^{-6} M) to the picomolar (10^{-12} M) range. For most protein antigens, initial concentration should be ~10 µg/ml, followed by nine 1:4 serial dilutions in blocking buffer. These antigen dilutions are assayed for their ability to inhibit the binding of conjugate to antigen-coated plates under standard assay conditions. From this initial assay, six 1:3 antigen dilutions spanning the dynamic range of inhibition are selected for further use as standard antigen-inhibitor dilutions. Prepare ≥75 µl of each dilution for each plate to be assayed.

Inhibitor curves are most sensitive in the region of the curve where small changes in inhibitor concentrations produce maximal changes in the amount of inhibition. This

region of the curve normally spans 15% to 85% inhibition. In most systems, this range of inhibition is produced by concentrations of inhibitor between 1 and 250 ng/ml.

4. Mix and incubate conjugate and inhibitor by adding 75 µl of 2× conjugate solution (from step 1) to each well of a round- or cone-bottom microtiter plate, followed by 75 µl inhibitor—either test antigen solution or standard antigen solution (from step 3). Mix the conjugate and inhibitor solutions by pipetting up and down in the pipet tip three times (see annotation to step 8 in the basic protocol) and incubate ≥30 min at room temperature.

   For accurate quantitation of the amount of antigen in the test solutions, test antigen solutions should inhibit conjugate binding between 15% to 85%. It may be necessary to assay two or three different dilutions of the test solutions to produce inhibitions within this range.

5. Prepare uninhibited control samples by mixing equal volumes of 2× conjugate solution and blocking buffer.

6. Transfer 50 µl of the mixture of conjugate plus inhibitor (from step 4) or conjugate plus blocking buffer (from step 5) to an antigen-coated plate (from step 2) and incubate 2 hr at room temperature.

   If samples are to be assayed in duplicate, the duplicates should be in adjacent columns on the same plate. Reserve column 11 for uninhibited control samples (step 5) and column 12 for substrate alone without any conjugate. If the concentration of antigen in the test samples is to be accurately quantitated, dilutions of homologous antigen solutions (step 3) should be included on each plate.

7. Wash plate as in steps 9 to 11 of the basic protocol.

8. Add 75 µl of MUP or NPP substrate solution to each well and incubate 1 hr at room temperature.

9. Read plates on the microtiter plate reader after ≥1 hr, at which time enough substrate has been hydrolyzed in the uninhibited reactions to permit accurate measurement of the inhibition.

10. Prepare a standard antigen-inhibition curve constructed from the inhibitions produced by the dilutions of the standard antigen solutions from step 3. Plot antigen concentration on the x axis, which is a log scale, and fluorescence or absorbance on the y axis, which is a linear scale.

11. Interpolate the concentration of antigen in the test solutions from the standard antigen-inhibition curve.

    The dynamic range of the inhibition curve may deviate from linearity if the specific antibodies are heterogeneous and possess significantly different affinities or if the standard antigen preparation contains heterogeneous forms of the antigen. Antigen concentration in test samples can be accurately interpolated from the inhibition curve as long as the test antigen is antigenically identical to the standard antigen and the concentration of test antigen falls within the dynamic range of inhibition.

ANTIBODY-SANDWICH ELISA TO DETECT SOLUBLE ANTIGENS

ALTERNATE PROTOCOL

Antibody-sandwich ELISAs may be the most useful of the immunosorbent assays for detecting antigen because they are frequently between 2 and 5 times more sensitive than those in which antigen is directly bound to the solid phase (Fig. 2.1.3). To detect antigen, the wells of microtiter plates are coated with specific (capture) antibody followed by incubation with test solutions containing antigen. Unbound antigen is washed out and a different antigen-specific antibody conjugated to enzyme (i.e., developing reagent) is added, followed by another incubation. Unbound conjugate is washed out and substrate is added. After another incubation, the degree of substrate hydrolysis is measured. The amount of substrate hydrolyzed is proportional to the amount of antigen in the test solution.

Additional Materials

Specific antibody or immunoglobulin fraction from antiserum or ascites fluid, *or* hybridoma supernatant (UNIT 2.6)

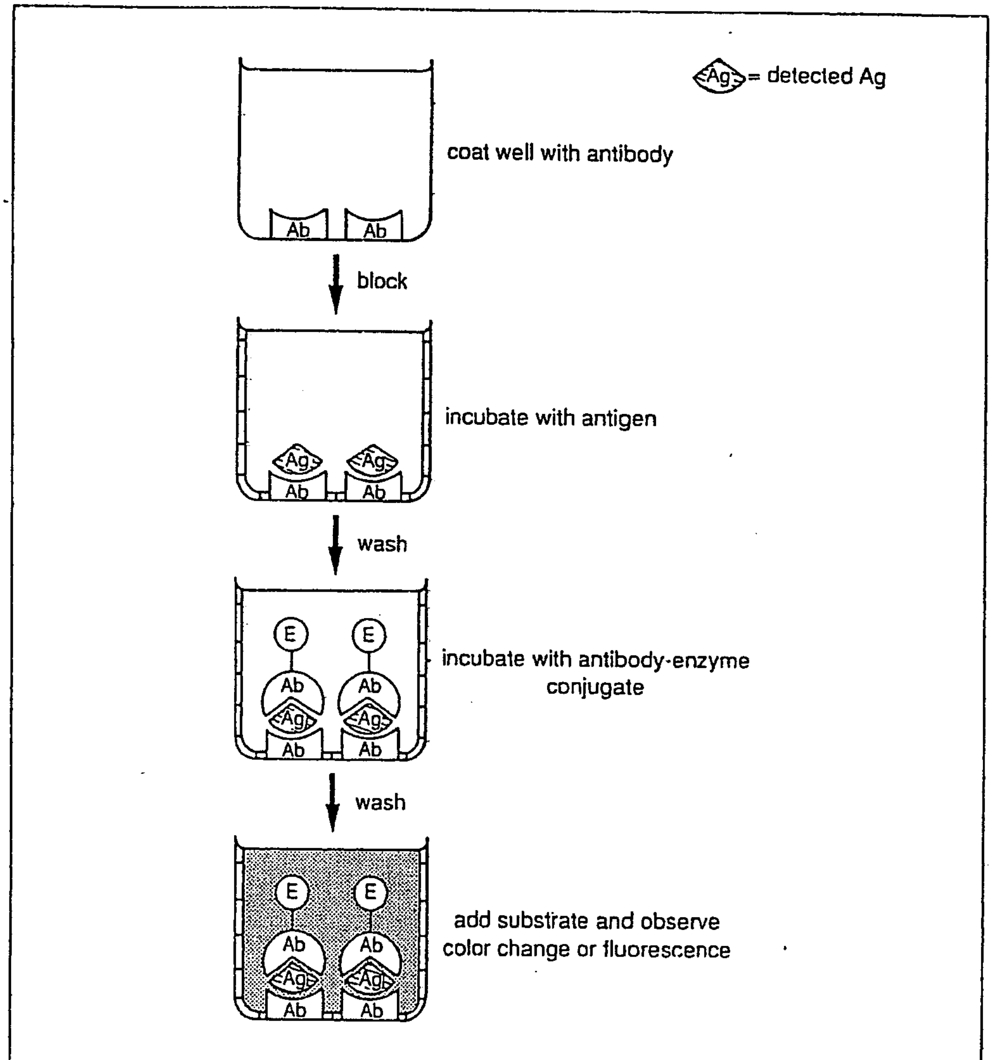

Figure 2.1.3 Antibody-sandwich ELISA to detect antigen. Ag = antigen; Ab = antibody; E = enzyme.

1. Prepare the capture antibody by diluting specific antibody or immunoglobulin fraction in PBSN to a final concentration of 0.2 to 10 μg/ml.

   The capture antibodies can be monoclonal or polyclonal.

   If the immunoglobulin fraction from an antiserum or ascites fluid is used, the concentration of total protein may need to be increased to compensate for the lower content of specific antibody. Little advantage is gained by increasing the total protein concentration in the capture antibody solution beyond 10 μg/ml.

2. Determine the concentration of capture antibody and conjugate necessary to detect the desired concentration of antigen by criss-cross serial-dilution analysis (see first support protocol). Prepare a capture antibody solution in PBSN at this concentration.

3. Coat wells of an Immulon plate with capture-antibody solution as in steps 3 to 5 of the basic protocol.

4. Block wells as in steps 6 and 7 of the basic protocol.

5. Prepare a standard antigen-dilution series by successive 1:3 dilutions of the homologous antigen stock in blocking buffer (see first support protocol).

   In order to measure the amount of antigen in a test sample, the standard antigen-dilution series needs to span most of the dynamic range of binding. This range typically spans from 0.1 to 1000 ng antigen/ml. The dynamic range of binding is defined as that range of antigen concentrations wherein small, incremental changes in antigen concentration produce detectable differences in the amount of antigen bound (see annotation to step 3, in the preceding alternate protocol). In most assay systems, the amount of antigen in a test solution is most accurately interpolated from the standard curve if it produces between 15% to 85% of maximal binding.

   Note: *While standard curves are necessary to accurately measure the amount of antigen in test samples, they are unnecessary for qualitative "yes/no" answers.*

6. Prepare dilutions of test antigen solutions in blocking buffer.

   It may be necessary to assay one or two serial dilutions of the initial antigen test solution to ensure that at least one of the dilutions can be accurately measured. For most assay systems, test solutions containing 1 to 100 ng/ml of antigen can be accurately measured.

7. Add 50-μl aliquots of the antigen test solutions and the standard antigen dilutions (from step 5) to the antibody-coated wells and incubate ≥2 hr at room temperature.

   For accurate quantitation, samples should be run in duplicate or triplicate, and the standard antigen-dilution series should be included on each plate (see step 5). Pipetting should be performed rapidly to minimize differences in time of incubation between samples.

8. Wash plate as in steps 9 to 11 of the basic protocol.

9. Add 50 μl specific antibody–alkaline phosphatase conjugate and incubate 2 hr at room temperature.

   The conjugate concentration is typically 25 to 400 ng specific antibody/ml.

   When the capture antibody is specific for a single determinant, the conjugate must be prepared from antibodies which recognize different determinants that remain available after the antigen is bound to the plate by the capture antibody.

10. Wash plate as in steps 9 to 11 of the basic protocol.

11. Add 75 μl of MUP or NPP substrate solution to each well and incubate 1 hr at room temperature.

12. Read the plate on a microtiter plate reader.

    To quantitate low-level reactions, the plate can be read again after several hours of hydrolysis.

13. Prepare a standard curve constructed from the data produced by serial dilutions of the standard antigen (step 5). Plot antigen concentration on the x axis which is a log scale, and fluorescence or absorbance on the y axis which is a linear scale.

14. Interpolate the concentration of antigen in the test solutions from the standard curve.

DOUBLE ANTIBODY–SANDWICH ELISA TO DETECT SPECIFIC ANTIBODIES

ALTERNATE PROTOCOL

This assay is especially useful when screening for specific antibodies in cases when a small amount of specific antibody is available and purified antigen is unavailable (Fig. 2.1.4). Additionally, this method can be used for epitope mapping of different monoclonal antibodies that are directed against the same antigen. Plates are coated with capture antibodies specific for immunoglobulin from the immunized species. The test antibody solution is incubated on the plates coated with the capture antibodies. Plates are then washed, incubated with antigen, washed again, and incubated with specific antibody conjugated to an enzyme. After incubation, unbound conjugate is washed out and substrate is added. Wells that are positive for hydrolysis may contain antibodies specific for the antigen.

Additional Materials

Capture antibodies specific for immunoglobulin from the immunized species
Specific antibody–alkaline phosphatase conjugate

1. Coat wells of an Immulon microtiter plate with 50 µl of 2 to 10 µg/ml capture antibodies as in steps 2 to 5 of the basic protocol.

   NOTE: *Capture antibodies must not bind the antigen or conjugate antibodies. When analyzing hybridoma supernatants or ascites fluid, coat plates with 2 µg/ml capture antibody. When analyzing antisera, coat plates with 10 µg/ml capture antibody.*

2. Block wells as in steps 6 and 7 of the basic protocol.

3. Prepare dilutions of test antibody solutions in blocking buffer. Add 50 µl to coated wells and incubate ≥2 hr at room temperature.

   Hybridoma supernatants, antisera, or ascites fluid can be used as the test samples. Dilute hybridoma supernatants 1:5 and antisera or ascites fluid 1:200.

4. Wash plate as in steps 9 to 11 of the basic protocol.

5. Prepare an antigen solution in blocking buffer containing 20 to 200 ng/ml antigen.

   Although purified antigen preparations are not essential, the limit of detectability for most protein antigens in this type of system is 2 to 20 ng/ml. A concentration of 20 to 200 ng antigen/ml is recommended.

6. Add 50-µl aliquots of the antigen solution to antibody-coated wells and incubate ≥2 hr at room temperature.

7. Wash plate as in steps 9 to 11 of the basic protocol.

8. Add 50 μl specific antibody–alkaline phosphatase conjugate to the wells and incubate 2 hr at room temperature.

The conjugate antibodies must not react with the capture antibody or the test antibody. The conjugate concentration is typically between 25 to 500 ng specific antibody/ml, and should be high enough to result in ~0.50 absorbance units/hr at 405 nm when using NPP as a substrate or a signal of 1000 to 1500 fluorescence units/hr when using MUP as a substrate. If no specific antibodies from the appropriate species

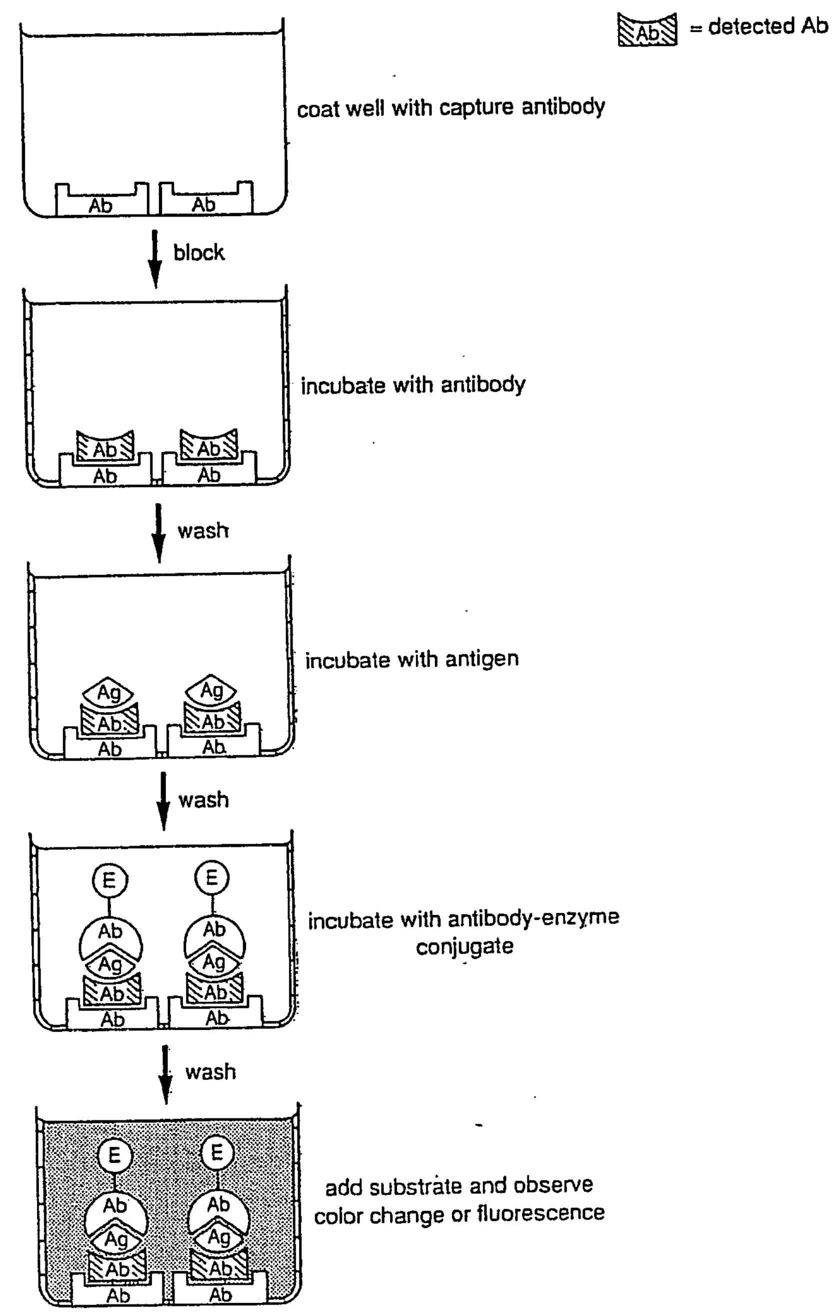

Figure 2.1.4 Double antibody-sandwich ELISA to detect specific antibodies. Ag = antigen; Ab = antibody; E = enzyme.

are available to serve as a positive control, then a positive control system should be constructed out of available reagents. Such reagents can be found in Linscott's Directory of Immunological and Biological Reagents.

9. Wash plate as in steps 9 to 11 of the basic protocol.

10. Add 75 µl of MUP or NPP substrate solution to each well and incubate 1 hr at room temperature. After 1 hr, examine hydrolysis visually or spectrophotometrically (see step 15 of the basic protocol).

    In order to detect low-level reactions, the plate can be read again after several hours or days of hydrolysis.

11. Check for false positives by rescreening samples that test positive for antigen-specific antibody. For each positive sample, coat four wells with capture antibody and arm the capture antibody with test antibody (steps 1 to 4). Incubate two of the wells with antigen (steps 5 to 7) and two of the wells with blocking buffer. Add conjugate and substrate to all four wells (steps 8 to 10) and measure hydrolysis after 1 hr.

    This procedure will eliminate false positives resulting from test antibodies that react with the enzyme-antibody complex.

ALTERNATE PROTOCOL

DIRECT CELLULAR ELISA TO DETECT CELL-SURFACE ANTIGENS

The expression of cell-surface antigens or receptors is measured using existing antibodies or other ligands specific for cell-surface molecules (Fig. 2.1.5). Cells are incubated with enzyme conjugated to antibodies that are specific for a cell-surface molecule. Unbound conjugate is washed away and substrate is added. The level of antigen expression is proportional to the amount of substrate hydrolysis. This procedure can be as sensitive as flow cytometry analysis in quantitating the level of antigen expression on a population of cells (*UNITS 5.1 - 5.4*). Unlike the flow cytometry analysis, however, this method is not sensitive for mixed populations. This assay can be converted to an indirect assay by substituting biotinylated antibody for the enzyme-antibody conjugate, followed by a second incubation with avidin–alkaline phosphatase.

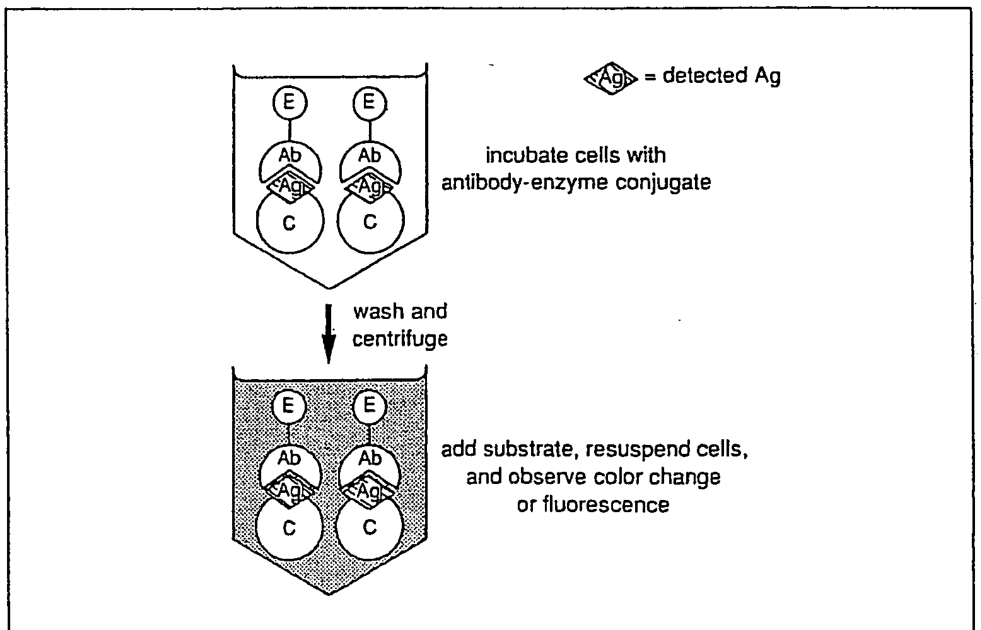

Figure 2.1.5 Direct cellular ELISA to detect cell-surface antigens. Ab = antibody; E = enzyme; C = cell.

Additional Materials

Cell samples

Specific antibody–alkaline phosphatase conjugate (see second support protocol)

Wash buffer, ice-cold

Cone- or round-bottom microtiter plates

Sorvall H-1000B rotor (or equivalent)

1. Determine the optimal number of cells per well and the antibody-conjugate concentration by criss-cross serial-dilution analysis (see first support protocol) using variable numbers of positive- and negative-control cell samples and varying concentrations of antibody-biotin conjugate.

   Titrate cells initially at 1-5 × 10^5/well and conjugate at 0.5 to 10 µg/ml. For preparation and handling of cells, consult steps 2 to 5.

   Because eukaryotic cells express variable amounts of alkaline phosphatase, test cells must be assayed in a preliminary experiment for alkaline phosphatase by incubation with substrate alone. If the test cells express unacceptable levels of alkaline phosphatase, another enzyme conjugate such as β-galactosidase should be used. Both chromogenic and fluorogenic substrates are available for β-galactosidase.

2. Centrifuge cell samples in a table-top centrifuge 5 min in Sorvall H-1000B rotor at 1500 rpm (450 × *g*), 4°C, in a 15- to 50-ml centrifuge tube. Count cells (APPENDIX 3) and resuspend in ice-cold wash buffer at 1-5 × 10^6 cells/ml.

   If the surface antigen retains its antigenicity after fixation, cells may be fixed at the beginning of the experiment—but do not fix cells unless it can be demonstrated that the antigenicity is retained after fixation. *Fix cells by suspending in glutaraldehyde (0.5% final; from a 25% stock, EM grade Sigma #G5882), and incubating 30 min at room temperature. Pellet cells, resuspend in PBSLE (see second support protocol), and incubate for 30 min at 37°C. Wash twice in PBSLE and resuspend in wash buffer. Cells can be kept for months at 4°C after fixation.*

3. Dispense 100 µl of cell suspension (1-5 × 10^5 cells) into wells of cone- or round-bottom microtiter plates, and centrifuge 1 min at 450 × *g*, 4°C. Remove supernatant by vacuum aspiration, and disrupt pellet by briefly shaking microtiter plate on a vortex mixer or microtiter plate shaker.

4. Resuspend pellet in 100 µl of conjugate in ice-cold wash buffer at the optimal concentration (see step 1). Incubate 1.5 hr at 4°C, resuspending cells by gently shaking at 15-min intervals.

   Be careful not to splash cell suspensions out of wells.

5. Centrifuge cells 1 min at 450 × *g*, 4°C, remove supernatant by vacuum aspiration, briefly vortex pellet, and resuspend in 200 µl ice-cold wash buffer. Repeat three times.

6. Add 100 µl MUP or NPP substrate solution. Incubate 1 hr at room temperature, resuspending cells by gently shaking at 15-min intervals during hydrolysis.

7. Determine extent of hydrolysis by visual inspection or using a microtiter plate reader.

INDIRECT CELLULAR ELISA TO DETECT ANTIBODIES SPECIFIC FOR SURFACE ANTIGENS

ALTERNATE PROTOCOL

This assay is designed to screen for antibodies specific for cell-surface antigens (Fig. 2.1.6). Antibodies against surface antigens are detected by incubating whole cells with a test solution containing the primary antibody. The unbound antibody is washed away and the cells are then incubated with an enzyme conjugated to antibodies specific for the primary antibody. Unbound enzyme conjugate is washed away and substrate solution added. The level of bound primary antibody is proportional to the amount of substrate hydrolysis.

Additional Materials

Positive-control antibodies (i.e., those that react with the experimental cells and are from the immunized species)
Negative-control antibodies (i.e., those that do not react with the experimental cells)
Test antibody solution
Antibody– or $F(ab')_2$ (against immunoglobulin from the immunized species)–alkaline phosphatase conjugate
Cone- or round-bottom microtiter plates

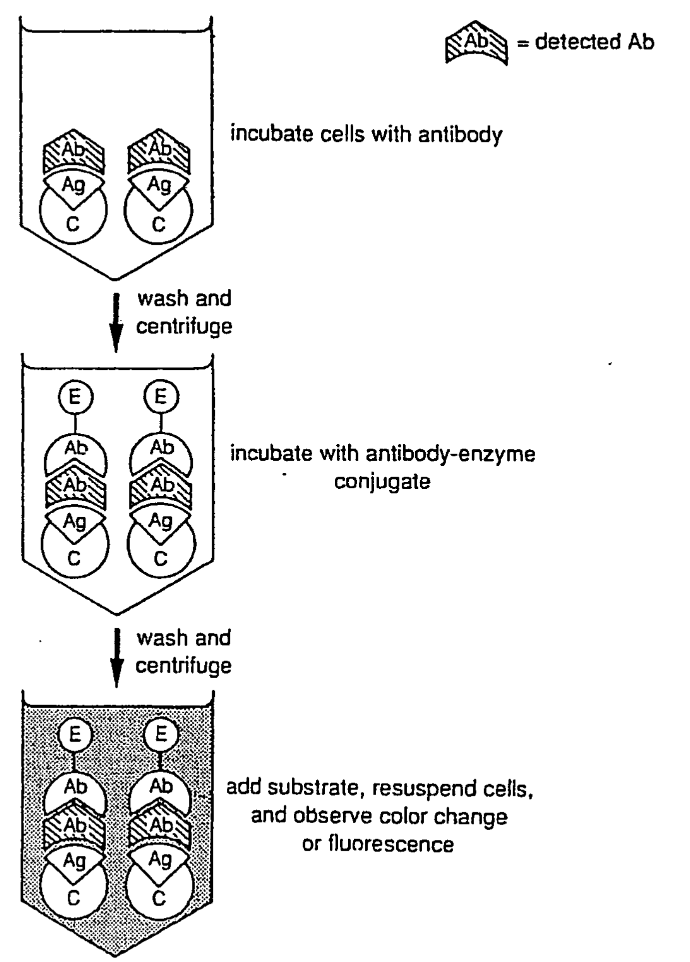

Figure 2.1.6 Indirect cellular ELISA to detect antibodies specific for surface antigens. Ab = antibody; E = enzyme; C = cell.

1. Centrifuge and resuspend cell samples as in step 2 of the previous alternate protocol at 1-5 × 10^6 cells/ml.

   Because this technique detects antibodies against uncharacterized epitopes, fixation prior to analysis is not recommended. Fixation may destroy the antigenicity of the epitope. All steps must be performed at 4°C in physiological buffers containing NaN_3.

   Because eukaryotic cells express variable amounts of alkaline phosphatase, test cells must be assayed for alkaline phosphatase activity. If the endogenous alkaline phosphatase level is too high, another enzyme should be substituted for alkaline phosphatase in the antibody-enzyme conjugate (see annotation to step 1 of the previous alternate protocol).

2. In preliminary assays, determine the optimal number of cells per well and conjugate concentration by criss-cross serial-dilution analysis using positive- and negative-control antibodies instead of test antibodies (see first support protocol). In adapting the criss-cross serial-dilution analysis, whole cells replace the solid-phase coating reagent; see techniques for handling cells are outlined in steps 3 to 8. Set up titrations by varying the number of cells between 1 × 10^5 and 5 × 10^5/well, the concentration of positive- and negative-control antibodies between 0.1 and 10 µg/ml, and the concentration of antibody-enzyme conjugate between 0.1 and 10 µg/ml.

3. Dispense 100 µl of cell suspension (1-5 × 10^5 cells) into wells of round- or cone-bottom microtiter plates. Centrifuge 1 min at 1500 rpm, 4°C, remove supernatant by vacuum aspiration, and disrupt pellet by briefly shaking microtiter plate on the vortex mixer.

4. Resuspend cells in 100 µl solutions containing 1 to 10 µg/ml test antibody or control antibodies in ice-cold wash buffer. Incubate 1.5 hr at 4°C, resuspending cells by gently shaking at 15-min intervals.

   Be careful not to splash cell suspensions out of wells.

5. Centrifuge cells 1 min at 1500 rpm, 4°C, remove supernatant by vacuum aspiration, briefly vortex pellet, and resuspend in 200 µl ice-cold wash buffer. Repeat twice.

6. Resuspend pellet in 100 µl enzyme-antibody conjugate or $F(ab')_2$-enzyme conjugate diluted in ice-cold wash buffer. The optimal concentration of antibody, determined in step 2, is usually 100 to 500 ng/ml. Incubate 1.5 hr at 4°C, resuspending cells by gently shaking at 15-min intervals.

   When working with cells that may express Fc receptors, it is best to use enzyme conjugated to $F(ab')_2$ fragments. $F(ab')_2$ fragments have had the Fc portion of the antibody enzymatically removed and no longer bind to Fc receptors.

7. Wash cells as in step 5. Repeat three times.

8. Add 100 µl MUP or NPP substrate solution. Allow hydrolysis to proceed until the signal has reached the desired levels; resuspend cells by gently shaking at 15 min intervals during hydrolysis. If desired, stop hydrolysis by adding 25 µl of 0.5 M NaOH.

9. Determine extent of hydrolysis by visual inspection or spectrophotometrically using a microtiter plate reader.

CRISS-CROSS SERIAL-DILUTION ANALYSIS TO DETERMINE OPTIMAL REAGENT CONCENTRATIONS

SUPPORT PROTOCOL

Serial dilution titration analyses are performed to determine optimal concentrations of reagents to be used in ELISAs. In this protocol, all three reactants in a three-step ELISA—a primary solid-phase coating reagent, a secondary reagent that binds the primary reagent, and an enzyme-conjugated tertiary developing reagent that binds to the secondary reagent—are serially diluted and analyzed by a criss-cross matrix analysis (Fig 2.1.7). Once the optimal concentrations of reagents to be used under particular assay conditions are determined, these variables are kept constant from experiment to experiment. The coating (primary), secondary, and developing (tertiary) reagents will vary depending upon which of the previous protocols needs to be optimized.

Additional Materials

Coating reagent
Secondary reagent
Developing reagent
17 × 100–mm and 12 × 74–mm test tubes

Secondary reactant

Tertiary reactant (antibody–alkaline phosphatase) (ng/ml)	homologous (antigen) 200	50	12.5	3.12	0.78	0	heterologous (antigen) 200	50	12.5	3.12	0.78	0	Rows
													A
500	over	over	over	3200	1000	0	500	120	40	20	10		B
250	over	over	over	2060	560	0	300	80	20	0	0		C
125	over	over	3650	1370	360	0	195	40	10	10	0		D
62.5	3600	4000	2270	790	240	0	120	30	10	10	10		E
31.25	2700	2100	1200	410	120	0	60	10	10	10	0		F
0	0	0	0	0	0	0	0	0	0	0	0		G
													H
Columns	1	2	3	4	5	6	7	8	9	10	11	12	

Figure 2.1.7 Results of a criss-cross serial-dilution analysis (for optimization of secondary and tertiary reactant concentrations) of an antibody-sandwich ELISA to detect antigen. The numbers in columns 1 to 11 and rows B to G represent relative fluorescence units observed for each well on a 96-well microtiter plate.

Plates were coated overnight with the capture antibody at 2 µg/ml. The secondary reactants, 4-fold serial dilutions of the homologous antigen and a non-cross-reactive heterologous antigen, were incubated on the plate 2 hr. The tertiary reactant, 2-fold serial dilutions of specific antibody–alkaline phosphatase conjugates, were incubated on the plate 2 hr. After 1 hr of incubation with the substrate MUP, the fluorescence was read in a microtiter plate spectrofluorometer.

Reagent concentrations depend upon individual assay variables that are set by the investigator. If the time of hydrolysis is set at 1 hr, the relative fluorescence at ~1000 relative fluorescence units, and the sensitivity at 780 pg/ml of homologous antigen, then 500 ng/ml of enzyme-antibody conjugate must be used in the ELISA. If, however, the assay has to detect only 3.12 ng/ml of homologous antigen, then the concentration of conjugate can be reduced to 125 ng/ml. It should be noted by comparing the homologous with the heterologous reactions (wells B5 versus B11 and D4 versus D10) that both the specificity and the signal-to-noise ratio for this assay are excellent.

Prepare coating-reagent dilutions

1. Place four 17 × 100–mm test tubes in a rack and add 6 ml PBSN to the last three tubes. In tube 1, prepare a 12-ml solution of coating reagent at 10 µg/ml in PBSN. Transfer 6 ml of tube 1 solution to tube 2. Mix by pipetting up and down five times. Repeat this transfer and mix for tubes 3 and 4; the tubes now contain the coating reagent at 10, 5, 2.5, and 1.25 µg/ml.

2. Using a multichannel pipet, dispense 50 µl of the coating reagent solutions into wells of four Immulon microtiter plates (i.e., each plate is filled with one of the four dilutions). Incubate overnight at room temperature or 2 hr at 37°C.

3. Rinse and block plates with blocking buffer as in steps 5 to 7 of the basic protocol.

Prepare secondary-reagent dilutions

4. Place five 12 × 75–mm test tubes in a rack and add 3 ml blocking buffer to the last four tubes. In tube 1, prepare a 4-ml solution of secondary reagent at 200 ng/ml in PBSN. Transfer 1 ml of tube 1 solution to tube 2. Pipet up and down five times. Repeat this transfer and mix for tubes 3 to 5; the tubes now contain the secondary reactant at 200, 50, 12.5, 3.125, and 0.78 ng/ml. If possible, prepare and test serial dilutions of a nonreactive heterologous form of the secondary reactant in parallel (Fig. 2.1.7).

   If the assay is especially insensitive, it may be necessary to increase the secondary reactant concentrations so the tube-1 solution is 1000 ng/ml.

5. Dispense 50 µl of the secondary reagent solutions into the first five columns of all four coated plates. The most dilute solution is dispensed into column 5, while solutions of increasing concentration are added successively into columns 4, 3, 2, and 1. Thus, the fifth column contains 0.78 ng/ml and the first column 200 ng/ml. Incubate 2 hr at room temperature.

6. Wash plates as in steps 9 to 11 of the basic protocol.

Prepare developing-reagent dilutions

7. Place five 17 × 100–mm test tubes in a rack and add 3 ml blocking buffer to the last four tubes. In tube 1, prepare a 6-ml solution of developing reagent at 500 ng/ml in blocking buffer. Transfer 3 ml of tube 1 solution into tube 2 and mix. Repeat this transfer and mixing for tubes 3 and 4—the tubes now contain the developing reagent at 500, 250, 125, 62.5, and 31.25 ng/ml.

8. Dispense 50 µl of the developing reagent solutions into the wells of rows 2 to 6 of each plate, dispensing the most dilute solution into row 6 and solutions of increasing concentration successively into rows 5, 4, 3, and 2. Incubate 2 hr at room temperature.

9. Wash plates as in steps 9 to 11 of the basic protocol.

Measure hydrolysis

10. Add 75 µl MUP or NPP substrate solution to each well, incubate 1 hr at room temperature, and measure the degree of hydrolysis visually or with a microtiter plate reader. An appropriate assay configuration results in 0.50 absorbance units/hr at 405 nm when using NPP as a substrate or 1000 to 1500 fluorescence units/hr when using MUP as a substrate.

    These results can be used to adjust optimal concentrations in the basic and alternate protocols.

PREPARATION OF ANTIBODY–ALKALINE PHOSPHATASE CONJUGATES

SUPPORT PROTOCOL

Antibodies are mixed with alkaline phosphatase and cross-linked by incubation with glutaraldehyde for 2 hr. The reaction is stopped by adding lysine and ethanolamine contained in PBSLE. The mixture is then desalted on a small Sephadex G-25 sizing column and the fractions are analyzed to detect those containing conjugate.

Additional Materials

>0.2 mg/ml antibody in PBS
Alkaline phosphatase in NaCl solution (Sigma #P0905)
25% glutaraldehyde, EM grade (Sigma #G5882)
PBS containing 100 mM lysine and 100 mM ethanolamine (PBSLE)
Blocking buffer containing 2.5 mM $MgCl_2$
10-ml Sephadex G-25 column (*APPENDIX 3*)
0.2-µm filter

1. Prepare a 1:3 mixture of antibody/alkaline phosphatase in PBS at >0.2 mg/ml total protein concentration.

   Because of the high specific activity and long shelf-life of most antibody–alkaline phosphatase conjugates, an initial preparation of 0.5 mg antibody and 1.5 mg alkaline phosphatase will usually produce enough conjugate to analyze 200 to 800 microtiter plates.

2. Add 25% glutaraldehyde to 0.2% final while vortexing. Incubate 2 hr at room temperature. Stop reaction by adding an equal volume of PBSLE.

3. Desalt the sample by chromatography on a Sephadex G-25 column in PBSN; bed volume of the column should be 5 to 10 times larger than the reaction volume. Collect fractions that are one-half the volume of the reaction volume.

4. Assay fractions by transferring 2 µl into tubes containing 0.5 ml NPP substrate solution. Pool the first five fractions that strongly hydrolyze NPP.

   While it is not essential to remove coupled from uncoupled reactants, this method will enrich for enzyme-antibody conjugates.

5. Mix the pool 1:2 in blocking buffer containing 2.5 mM $MgCl_2$, filter through a 0.2-µm filter, and store at 4°C.

REAGENTS AND SOLUTIONS

Borate-buffered saline (BBS)

0.17 M H_3BO_4
0.12 M NaCl
Adjust to pH 8.5 with NaOH

Blocking buffer

BBS (see above) containing:
0.05% Tween 20
1 mM EDTA
0.25% bovine serum albumin (BSA)
0.05% NaN_3
Store at 4°C

Gelatin may be substituted for BSA; 5% instant milk has been successfully used but may interfere nonspecifically with antibody binding.

MUP substrate solution

0.2 mM 4-methylumbelliferyl phosphate (MUP; Sigma #M8883)
0.05 M $NaCO_3$
0.05 mM $MgCl_2$
Store at room temperature

NPP substrate solution

3 mM *p*-nitrophenyl phosphate (NPP; Sigma #104-0)
0.05 M $NaCO_3$
0.05 mM $MgCl_2$
Store at 4°C

Test antibody solution

Hybridoma supernatants (*UNIT 2.6*) can usually be diluted 1:5 and ascites fluid and antisera (UNIT 2.4) diluted 1:500 in blocking buffer and still generate a strong positive signal. Dilutions of nonimmune ascites or sera should be assayed as a negative control. Prepare antibody dilutions in cone- or round-bottom microtiter plates before adding them to antigen-coated plates.

Sources of appropriate antibodies and conjugates can be found in Linscott's Directory of Immunological and Biological Reagents.

Test antigen solution

0.2 to 10 µg/ml antigen, purified or partially purified in PBSN; store at 4°C

Wash buffer

Hanks balanced salt solution (HBSS; *APPENDIX 2*)
1% fetal calf serum (FCS; heat-inactivated 60 min, 56°C)
0.05% NaN_3
Store at 4°C

COMMENTARY

Background Information

Since their first description in 1971 (Engvall and Perlman), ELISAs have become the system of choice when assaying soluble antigens and antibodies. Factors that have contributed to their success include their sensitivity, the long shelf-life of the reagents (alkaline phosphatase conjugates typically lose only 5% to 10% of their activity per year), the lack of radiation hazards, the ease of preparation of the reagents, the speed and reproducibility of the assays, and the variety of ELISA formats that can be generated with a few well-chosen reagents. Additionally, no sophisticated equipment is necessary for many ELISA applications, including screening hybridoma supernatants for specific antibodies and screening biological fluids for antigen content.

The ELISAs described here combine the special properties of antigen-antibody interactions with simple phase separations to produce powerful assays for detecting biological molecules. The multivalency of antibodies can result in the formation of long-lived antigen-antibody complexes, thus allowing long periods of time during which such complexes can be measured. By designing an assay so that a capture reagent initiates the binding of antigen-antibody complexes and enzyme conjugates onto a solid phase, the unbound reagents can be easily and rapidly separated from the solid phase. The solid phase is washed and the amount of bound conjugate is visualized by incubating the solid phase with a substrate that forms a detectable product when hydrolyzed by the bound enzyme. ELISAs are similar in principle to radioimmunoassays, except that the radioactive label is replaced by an enzyme conjugate.

A number of different enzymes have been successfully used in ELISAs, including alkaline phosphatase, horseradish peroxidase, β-galactosidase, glucoamylase, and urease. Alkaline phosphatase—perhaps the most widely used conjugated enzyme—is recommended because of its rapid catalytic rate, excellent intrinsic stability, availability, ease of conjugation, and resistance to inactivation by common laboratory reagents. Additionally, the substrates of alkaline phosphatase are nontoxic

Double-Immunodiffusion Assay for Detecting Specific Antibodies

UNIT 2.3

BASIC PROTOCOL

Double immunodiffusion is a simple gel-based assay for detecting antigen-specific antibodies. Analytical agar gels are poured onto microscope slides that have been precoated with agar. Small wells are punched 0.5- to 0.75-cm apart in the analytical gel. Antigen and antibody solutions are placed in adjacent wells and allowed to diffuse into the gel for 6 to 48 hr. As antibody and antigen form diffusion gradients that cross each other, a line of immunoprecipitation may form between the wells, indicating the presence of specific antibodies. The gel is then stained and destained until precipitin lines are maximally visible.

Materials

Noble agar (Difco)
PBS (*APPENDIX 2*) containing 0.05% NaN_3 (PBSN)
4% PEG 6000 (J.T. Baker) in PBSN, prewarmed to 56°C (store at room temperature)
1 mg/ml antigen
Antisera
Staining solution
Destaining solution

2 × 3–in. microscope slides, precleaned
Boiling and 56°C water baths
50°C oven
Template (see Fig. 2.3.1)
15-G stainless steel needle (blunt-ended and beveled) *or* immunodiffusion punch set (EC Apparatus)
10-µl Hamilton syringe
Humidified chamber (enclosed plastic container with moistened tissues; Fig. 3.8.1)
Staining rack and dish
Whatmann 3MM filter paper

Precoat microscope slides with agar

1. Prepare a 0.5% noble agar solution in PBSN and place in a boiling water bath until agar dissolves.

2. Place 2 × 3–in. microscope slides on a level surface and pipet 8 ml of 0.5% melted agar evenly over the surface of each slide. Do not disturb gels until the agar has set.

3. Allow gels to dry 4 hr in a 50°C oven or overnight at room temperature.

   A dried agar precoat provides an adhesive base that prevents the analytical agar from separating from the slide during staining and destaining treatments.

Prepare analytical gel

4. Dissolve 2% noble agar in PBSN in a boiling water bath.

5. Place coated microscope slides on a level surface. Cool the 2% melted agar to 56°C. Mix 2% melted agar 1:1 with 56°C PEG solution and pipet 10 ml evenly over each slide. Do not disturb gels until the agar has set.

   PEG stabilizes immunoprecipitates and increases their visibility.

6. Place the agar gel over a template and, using a blunt-ended and beveled 15-G needle or an immunodiffusion punch set, carefully punch wells to accommodate all antigen and antisera solutions to be tested (see Fig. 2.3.1).

7. Remove agar plugs using a Pasteur pipet attached to a vacuum line.

   Use a weak vacuum to remove the agar plugs, taking care not to disturb the surrounding agar field.

Load the gel

8. Prepare three antigen samples to be tested against undiluted antisera—one should be ~1 mg/ml, and the other two should be ~500 and 250 μg/ml (prepared by diluting 1 mg/ml antigen 1:1 serially with PBSN. Using a 10-μl Hamilton syringe or pipettor, fill the central wells with an antigen sample and surrounding wells with antisera (the wells hold 5 to 10 μl). Maintain slides on a level surface, and allow samples to diffuse into the gel.

   When screening antisera, multiple dilutions of antigen should be tested against all antisera. To increase the amount of reagent loaded, wells can be filled 2 or 3 times. After the liquid is absorbed into the gel (~5 to 10 min), the wells may be refilled with antigen solution.

9. Place loaded gels in a humidified chamber and incubate 48 hr at room temperature. Examine the gels and score for precipitin lines at 6, 24, and 48 hr.

   Gels should not be in direct contact with the moistened tissues in the humidity chamber.

Wash and stain the gels

10. Place gels in a staining rack. Place rack in a staining dish filled with PBSN and incubate 24 hr at room temperature with gentle stirring using a magnetic stirbar.

    Washing is done in steps 10 to 12 to remove proteins that are not precipitated.

11. Replace PBSN with fresh solution and incubate 24 hr at room temperature.

12. Remove salt by replacing PBSN with water. Incubate 4 hr at room temperature.

13. Remove gels from the staining rack and place face-up on a flat surface. Dry the gels by covering with 3MM filter paper and leaving overnight at room temperature.

14. Place dry gels in a staining rack and immerse 10 min in staining solution at room temperature.

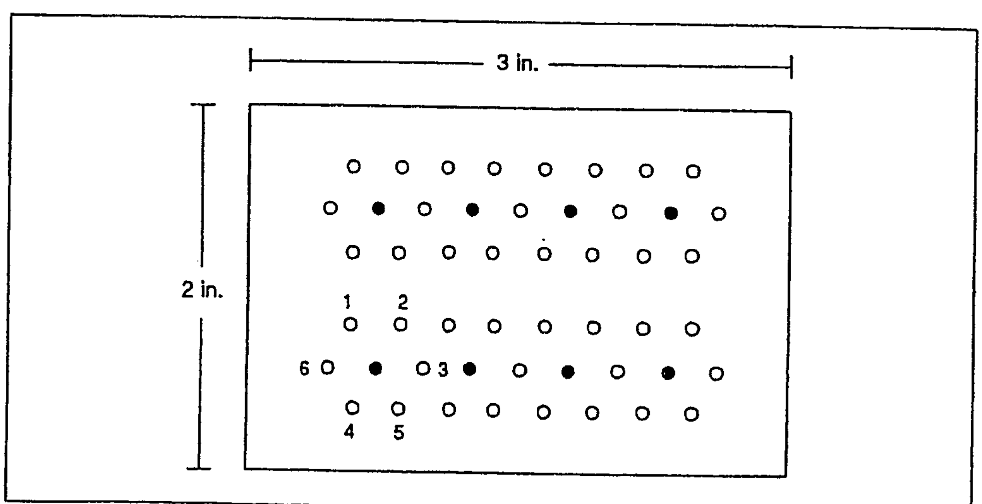

Figure 2.3.1 Double-diffusion template consisting of eight partially overlapping hexagonal arrays distributed around eight central wells. Central wells are represented by dark circles. A numbered set of wells arranged hexagonally around a central well is shown.

15. Destain by immersing gels 4 min in destaining solution. Repeat until precipitin lines are maximally visible and the background staining is negligible.

16. Air dry the gels at room temperature.

REAGENTS AND SOLUTIONS

Destaining solution

15% (vol/vol) ethanol
5% (vol/vol) glacial acetic acid
80% H_2O
Store at room temperature

Staining solution

0.5% (wt/vol) Coomassie Brilliant Blue R-250
40% (vol/vol) ethanol
10% (vol/vol) glacial acetic acid
50% H_2O
Store at room temperature

COMMENTARY

Background Information

Gel-diffusion techniques, among the earliest methods for detecting specific antibodies and for measuring antigenicity (Ouchterlony and Nilsson, 1986), are still useful methods for detecting specific antibodies. However, they do require high concentrations of both antigen and antibody and are relatively insensitive to antibodies with low affinities. Recently, a silver-staining technique has been described that increases the sensitivity of double-diffusion assays 10 to 100 times (Rochu et al., 1989).

Double immunodiffusion owes its success to the unique nature of antibody-antigen interactions. When polyvalent antibodies with moderate-to-high intrinsic affinities are mixed with antigen at the right ratio—called the zone of equivalence—lattices of antibody-antigen complexes form and precipitate out of solution. When gradients of antigen and antibody are established by diffusion from adjacent wells in a bed of agar, a line of practically insoluble precipitation forms at the equivalence zone (precipitin lines).

Critical Parameters

In this assay the initial antigen and antibody concentrations must be able to support the formation of equivalence zones. For this reason, three different antigen concentrations are recommended. If no lines of precipitation are observed, more sensitive techniques, (e.g., ELISAs) should be considered.

Anticipated Results

Double-diffusion assays in which the immunoprecipitates are stained with Coomassie Brilliant Blue can be sensitive to as little as 25 µg/ml of specific antibody. In the absence of staining, the assay is sensitive to ~100 µg/ml of specific antibody. Details of the precipitation patterns (double precipitin lines, spurs, and lines of identity) can reveal information about the antigenic specificities of various antisera and information about the structure of the antigen (Ouchterlony and Nilsson, 1986). Figure 2.3.2 illustrates typical results.

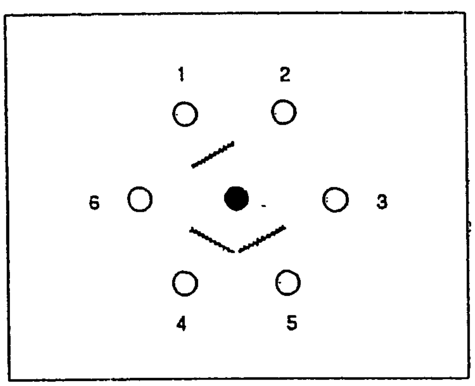

Figure 2.3.2 Typical results of a double-immunodiffusion assay. Wells 1, 4, and 5 are positive for reactive antisera, while wells 2, 3, and 6 are negative.

Time Considerations

The higher the titer of specific antibody, the more rapid the precipitation. Precipitation may begin in some systems within a few hours, while in others it may take 24 to 48 hr to complete.

Literature Cited

Ouchterlony, O. and Nilsson, L-A. 1986. Immunodiffusion and immunoelectrophoresis. *In* Handbook of Experimental Immunology, Vol. 1: Immunochemistry (D.M. Weir, L.A. Herzenberg, C. Blackwell, and L.A. Herzenberg, eds.) pp. 32.1-32.50. Blackwell, Oxford.

Rochu, D., Crespeau, H., Fine, A., and Fine, J-M. 1989. A sensitive double-diffusion microassay suitable for the detection of idiotype–anti-idiotype precipitates. *J. Immunol. Methods* 118:67-71.

Key Reference

Ouchterlony and Nilsson, 1986. See above.

Contains a detailed description of immunodiffusion techniques and provides detailed interpretations of various patterns of immunoprecipitation observed in double-immunodiffusion experiments.

Contributed by Peter Hornbeck
University of Maryland
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Crossreactivity of VEGF-2 antibodies